THE IN VITRO CHARACTERIZATION OF THE DRUG-PROTEIN BINDING OF RACEMIC PROPAFENONE, AND ITS ACTIVE METABOLITE 5-HYDROXYPROPAFENONE IN HUMAN SERUM, AND IN SOLUTIONS OF ISOLATED HUMAN SERUM PROTEINS.

by

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We accept this thesis as conforming to the required standard

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Department of PHARMACEUTICAL SCIENCES

The University of British Columbia
Vancouver, Canada

Date October 10, 1990
ABSTRACT

An accurate plasma concentration-response relationship for propafenone (PF), a potent class 1 antiarrhythmic agent, has not yet been defined. A general pharmacological premise suggests that only the free drug is available to contribute to the observed pharmacological response. It has previously been shown that PF is highly bound to α-1-acid glycoprotein (AAG) which results in a low free PF concentration. The correlation of free PF concentration and response failed to adequately describe the dose response relationship. It has subsequently been shown that upon chronic dosing, two active metabolites, namely 5-hydroxypropafenone (5-OH-PF), and n-depropylpropafenone (n-depropyl-PF) accumulate in humans treated with PF. It is highly likely that the free concentration of PF, in addition to those of 5-OH-PF and n-depropyl-PF, contributes to the observed pharmacological effect following administration of PF at steady-state. To date, no accurate estimation of 5-OH-PF binding in serum has been established. This thesis examines the binding characteristics of PF and 5-OH-PF and their interaction in human serum, and in solutions of AAG, human serum albumin (HSA), high density lipoproteins (HDL), low density lipoproteins (LDL), and very low density lipoproteins (VLDL) using equilibrium dialysis.

The binding of PF (2.0 µg/mL) and 5-OH-PF (0.5 µg/mL) was examined in serum when both drug and metabolite were present. The free fraction (FF) of PF and 5-OH-PF in serum was 0.063 ± 0.004 and 0.232 ± 0.020, respectively. Both PF and 5-OH-PF were found to bind to a high affinity, low capacity binding site on AAG, in addition PF showed a
second low affinity, high capacity binding site. PF displayed a 10 fold greater affinity for the high affinity binding site on AAG when compared to 5-OH-PF. Both PF and 5-OH-PF showed only one low affinity, high capacity site on HSA of similar affinity. The interaction of PF and 5-OH-PF with HDL, LDL, and VLDL appeared to be due to solubilization, rather than a "true" drug-protein binding interaction, since it correlated well with the concentration of cholesterol within the lipoprotein complex (PF, r^2=0.85; 5-OH-PF, r^2=0.96). However, PF appeared to show saturable binding to the HDL complex. The uptake of PF and 5-OH-PF was greatest in LDL followed by HDL, and finally VLDL. In serum PF displayed both a high affinity, low capacity, and a low affinity, high capacity binding sites, although a similar observation was expected for 5-OH-PF, only one binding site could be experimentally identified.

The uptake of 5-OH-PF by red blood cells (RBC) appeared to be approximately 5 fold greater than that of PF (i.e. The ratio of PF and 5-OH-PF concentration in the red blood cell/plasma was 0.7 ± 0.1 and 3.2 ± 0.5, respectively).

When the binding of PF and 5-OH-PF was considered separately, the binding profiles were similar, that is, both drugs showed high affinity binding to AAG, and low affinity binding and/or non-specific binding to other serum proteins such as HSA, HDL, LDL, and VLDL. However, when both drug and metabolite were present, the binding of 5-OH-PF to AAG was found to be reduced. This is thought to occur as a result of the displacement of 5-OH-PF by PF from AAG. Thus, the binding of 5-OH-PF
was noted to be more dependent on HSA, and lipoproteins when compared to PF. On the other hand, the binding of PF (2.0 μg/mL), even with the addition of 5-OH-PF, was dependent largely on the concentration of AAG. Although the binding of 5-OH-PF was apparently not altered by the addition of PF in serum, a decrease in the binding of 5-OH-PF by the addition of PF was observed. It is hoped that the understanding gained from this thesis will provide information regarding the relative importance of free PF and 5-OH-PF plasma concentration in future pharmacodynamic studies of PF.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>ii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xvii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xviii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xx</td>
</tr>
</tbody>
</table>

1. INTRODUCTION 1

1.1 PROPAFENONE 1

1.1.1 PHARMACOLOGY OF PROPAFENONE 1
1.1.2 THERAPEUTIC EFFICACY OF PROPAFENONE 2
1.1.3 PHARMACOKINETICS OF PROPAFENONE 3

1.1.3.1 ABSORPTION OF PROPAFENONE 3
1.1.3.2 DISTRIBUTION AND PLASMA PROTEIN BINDING OF PROPAFENONE 3
1.1.3.3 METABOLISM OF PROPAFENONE 4

1.2 DRUG-PROTEIN BINDING 7

1.2.1 ULTRAFILTRATION 9
1.2.2 EQUILIBRIUM DIALYSIS 10

1.3 SERUM PROTEINS 12

1.3.1 HUMAN SERUM ALBUMIN 13
1.3.2 α-1-ACID GLYCO PROTEIN 14
1.3.3 SERUM LIPOPROTEINS 15
1.3.4 UPTAKE OF DRUGS INTO RED BLOOD CELLS 16
1.3.5 CHEMICAL BASIS FOR DRUG-PROTEIN INTERACTION 17

1.4 QUANTITATIVE DESCRIPTION OF THE DRUG-PROTEIN BINDING SITE 18

1.5 RATIONALE 22

1.6 AIMS 25
2. EXPERIMENTAL

2.1 MATERIALS AND SUPPLIES

2.1.1 DRUGS, METABOLITES, AND INTERNAL STANDARDS
2.1.2 CHEMICALS AND REAGENTS
2.1.3 PROTEINS
2.1.4 SOLVENTS
2.1.5 GASES
2.1.6 EQUILIBRIUM DIALYSIS CELLS
2.1.7 ULTRAFILTRATION DEVICES
2.1.8 ELECTROPHORESIS

2.1.8.1 POLYACRYLAMIDE ELECTROPHORESIS GELS
2.1.8.2 AGAROSE ELECTROPHORESIS GELS

2.1.9 RADIAL IMMUNO DIFFUSION PLATES
2.1.10 OTHER SUPPLIES

2.2 EQUIPMENT

2.2.1 GAS LIQUID CHROMATOGRAPH
2.2.2 SPECTROPHOTOMETER
2.2.3 ELECTROPHORESIS EQUIPMENT
2.2.4 OTHER

2.3 PREPARATION OF STOCK AND REAGENT SOLUTIONS

2.3.1 DRUGS, METABOLITES, AND INTERNAL STANDARDS
2.3.2 REAGENTS AND SOLUTIONS
2.3.3 PROTEIN SOLUTIONS
2.3.4 ESTABLISHED TECHNIQUES USED IN RESEARCH

2.3.4.1 GAS LIQUID CHROMATOGRAPHY OF PROPafenONE AND 5-HYDROXYPROPafenONE
2.3.4.2 LowRY PROTEIN ASSAY
2.3.4.3 POLYACRYLAMIDE GEL ELECTROPHORESIS
2.3.4.4 LIPOPROTEIN AGAROSE ELECTROPHORESIS

2.4 PROTEIN BINDING OF 5-HYDROXYPROPafenONE AND PROPafenONE

2.4.1 PRELIMINARY EXPERIMENTS

2.4.1.0 PREPARATION OF EQUILIBRIUM DIALYSIS DEVICES
2.4.1.1 TIME TO EQUILIBRIUM
2.4.2.2 Non-specific binding of 5-hydroxypropafenone to equilibrium dialysis cells and membranes. 41
2.4.2.3 Non-specific binding of propafenone and 5-hydroxypropafenone to Amicon MPS-1 ultrafiltration devices. 41
2.4.3 pH dependent binding of propafenone and 5-hydroxypropafenone 41
2.4.3.1 pH dependent binding of propafenone in serum 41
2.4.3.2 pH dependent binding of propafenone and 5-hydroxypropafenone in isolated protein solutions (HSA and AAG) 42
2.4.3.3 pH dependent partitioning in toluene and hexane of propafenone and 5-hydroxypropafenone. 42
2.4.4 Buffer strength 43
2.4.5 Chemical degradation of 5-hydroxypropafenone during dialysis 43
2.4.6 Volume shifts 44
2.4.7 Osmolarity of 5-hydroxypropafenone solutions 44
2.4.8 Loss of protein during dialysis 44
2.4.9 Test for dialysis membrane integrity following dialysis 45
2.5 Binding of 5-hydroxypropafenone in human serum 45
2.5.1 Binding in normal serum 45
2.5.2 Rosenthal plots of 5-hydroxypropafenone binding in serum 46
2.5.3 Protein binding of 5-hydroxypropafenone in serum determined by equilibrium dialysis in the presence of propafenone 46
2.5.4 Binding of propafenone and 5-hydroxypropafenone using the method of ultrafiltration 47
2.6 Binding of 5-hydroxypropafenone and propafenone to isolated proteins 47
2.6.1 Binding of propafenone and 5-hydroxypropafenone to HSA and AAG 47
2.6.2 Binding to propafenone and 5-hydroxypropafenone to albumin and free fatty acid free albumin. 48
2.6.3 Binding of propafenone and 5-hydroxypropafenone to lipoprotein deficient serum 49
2.6.4 Electrophoresis of isolated protein solutions 49
2.6.5 Drug-drug displacement of propafenone and 5-hydroxypropafenone from serum proteins by disopyramide, and ibuprofen 50
2.6.6 Drug-drug displacement of propafenone and 5-hydroxypropafenone from HSA and AAG by disopyramide and ibuprofen 50
2.7 Characterization of binding of propafenone and 5-hydroxypropafenone in protein solutions 51
2.7.1 Characterization of binding to pooled human serum, AAG, human albumin, HDL, LDL, and VLDL solutions 51
2.7.2 Electrophoresis of lipoproteins 52
2.8 Data analysis 52
2.8.1 Statistical analysis of data 52
2.8.2 Binding parameter models and data fitting 53
2.8.3 Mathematical binding reconstitution studies 54
2.9 Red blood cell distribution 55
2.9.1 Preparation of red blood cells 56
2.8.2 Time to distribution equilibrium for propafenone and 5-hydroxypropafenone uptake into human red blood cells 56
2.8.3 Uptake of propafenone and 5-hydroxypropafenone by human red blood cells. 57

3. Results 58
3.1 Preliminary in vitro protein binding experiments for propafenone and 5-hydroxypropafenone 58
3.1.1 Time to equilibrium 58
3.1.2 Non-specific binding of propafenone and 5-hydroxypropafenone to equilibrium dialysis cells and MPS-1 ultra-filtration devices 58
3.1.3 pH dependent binding of propafenone and 5-hydroxypropafenone. 61
3.1.3.1 pH dependent binding of propafenone in human serum, and solutions of human serum albumin, and human alpha-1-acid glycoprotein. 61
3.1.3.2 pH DEPENDENT BINDING
5-HYDROXYPROPafenONE
IN HUMAN SERUM, A SOLUTION OF
HUMAN SERUM ALBUMIN, AND OF HUMAN
ALPHA-1-ACID GLYCOProTEIN. 61

3.1.3.3 THE INFLUENCE OF pH ON THE
PARTITIONING OF PROPafenONE AND
5-HYDROXYPROPafenONE IN A
TOLuENE PHOSPHATE BUFFER AND HEXANE
PHOSPHATE BUFFER SYSTEM. 64

3.1.4 THE EFFECT OF BUFFER STRENGTH ON BINDING OF
5-HYDROXYPROPafenONE. 64

3.1.5 DEGRADATION OF 5-HYDROXYPROPafenONE
DURING EQUILIBRIUM DIALYSIS. 68

3.1.6 VOLUME SHIFTS 68

3.1.7 OSMOLLARITY OF 5-HYDROXYPROPafenONE STOCK
SOLUTIONS USED IN EQUILIBRIUM DIALYSIS
STUDIES. 69

3.1.8 PROTEIN LOSS DURING EQUILIBRIUM DIALYSIS 69

3.1.9 TEST FOR DIALYSIS MEMBRANE INTEGRITY
FOLLOWING DIALYSIS 69

3.2 PROTEIN BINDING OF 5-HYDROXYPROPafenONE IN SERUM
OF HEALTHY MALE VOLUNTEERS 70

3.2.1 PROTEIN BINDING OF THE METABOLITE,
5-HYDROXYPROPafenONE, IN THE PRESENCE OF
THE PARENT COMPOUND PROPafenONE AT A
THERAPEUTIC CONCENTRATION AS DETERMINED BY
EQUILIBRIUM DIALYSIS AND ULTRAFILTRATION. 75

3.3 BINDING PROFILES OF PROPafenONE AND
5-HYDROXYPROPafenONE TO ISOLATED HUMAN SERUM
PROTEINS, LIPOPROTEIN DEFICIENT SERUM, AND NORMAL
SERUM. 76

3.3.1 BINDING OF PROPafenONE AND
5-HYDROXYPROPafenONE TO ISOLATED HUMAN
SERUM PROTEINS. 76

3.3.2 BINDING OF PROPafenONE AND
5-HYDROXYPROPafenONE TO PURE ISOLATED
HUMAN SERUM PROTEINS DISSOLVED
IN EITHER BUFFER OR SERUM ULTRAFILTRATE. 78

3.3.3 THE BINDING OF PROPafenONE AND
5-HYDROXYPROPafenONE TO FREE FATTY ACID FREE
ALBUMIN COMPARED TO NORMAL ALBUMIN. 81

3.3.4 BINDING OF PROPafenONE AND
5-HYDROXYPROPafenONE TO LIPOPROTEIN
DEFICIENT SERUM VS. NORMAL SERUM 81

3.3.5 DISPLACEMENT OF PROPafenONE AND
5-HYDROXYPROPafenONE FROM PROTEIN BINDING
SITES BY IBUPROFEN AND DISOPYRAMIDE. 84
3.3.6 DISPLACEMENT OF PROPafenONE AND 5-HYDROXYPROPafenONE FROM ISOLATED HUMAN SERUM PROTEINS BY DISOPYRAMIDE AND IBUPROFEN

3.3.7 PURITY ASSURANCE OF ISOLATED PROTEIN IN BINDING EXPERIMENTS

3.4 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE.

3.4.1 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN ALPHA-1-ACID GLYCOPROTEIN SOLUTION.

3.4.2 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN HUMAN SERUM ALBUMIN SOLUTION

3.4.3 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN POOLED HUMAN SERUM

3.4.4 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN A SOLUTION OF HIGH DENSITY LIPOPROTEINS.

3.4.5 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN A SOLUTION OF LOW DENSITY LIPOPROTEINS.

3.4.6 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN A SOLUTION OF VERY LOW DENSITY LIPOPROTEINS.

3.4.7 MATHEMATICAL BINDING EQUATIONS FOR PROPafenONE AND 5-HYDROXYPROPafenONE.

3.4.8 PROTEIN QUALITY ASSURANCE DURING BINDING CHARACTERIZATION EXPERIMENTS

3.5 UPTAKE OF PROPafenONE AND 5-HYDROXYPROPafenONE INTO RED BLOOD CELLS

3.5.1 TIME TO EQUILIBRIUM FOR RED BLOOD CELL UPTAKE OF PROPafenONE AND 5-HYDROXYPROPafenONE.

3.5.2 UPTAKE OF PROPafenONE AND 5-HYDROXYPROPafenONE BY RED BLOOD CELLS

4. DISCUSSION

4.1.1 PROTEIN BINDING AND PROPafenONE

4.1.2 EQUILIBRIUM DIALYSIS TIME TO EQUILIBRIUM

4.1.3 NON-SPECIFIC BINDING OF PF AND 5-HYDROXYPROPafenONE

4.1.4 pH DEPENDENT BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE

4.1.5 THE EFFECT OF BUFFER STRENGTH ON THE DRUG PROTEIN BINDING OF 5-HYDROXYPROPafenONE.
4.1.6 Degradation of 5-Hydroxypropafenone During Equilibrium Dialysis 114
4.1.7 Volume Shifts During Equilibrium Dialysis of Propafenone and 5-Hydroxypropafenone. 114
4.1.8 Osmolarity Measurements of 5-Hydroxypropafenone Drug Solutions. 115
4.1.9 Protein Loss During Equilibrium Dialysis of Propafenone and 5-Hydroxypropafenone 115
4.1.10 Membrane Integrity During Equilibrium Dialysis 116

4.2 Protein Binding of Propafenone and 5-Hydroxypropafenone 117
4.2.1 Protein Binding of 5-Hydroxypropafenone in Serum of Healthy Males 117
4.2.2 Protein Binding of Propafenone and 5-Hydroxypropafenone in Serum Estimated by Equilibrium Dialysis and Ultrafiltration. 124

4.3 Binding of Propafenone and 5-Hydroxypropafenone to Isolated Serum Proteins 126
4.3.1 Binding Differences Between Propafenone and 5-Hydroxypropafenone in Various Protein Solutions. 126
4.3.2 The Effect of Buffer and Serum Ultrafiltrate on the Binding of Propafenone and 5-Hydroxypropafenone. 129
4.3.3 The Effect of the Removal of Free Fatty Acids from Albumin on the Binding of Propafenone and 5-Hydroxypropafenone. 130
4.3.4 The Effect of the Removal of Lipoproteins from Serum on the Binding of Propafenone and 5-Hydroxypropafenone. 131
4.3.5 Displacement of Propafenone and 5-Hydroxypropafenone in Serum by Displacing Agents Ibuprofen and Disopyramide. 132
4.3.6 Displacement of Propafenone and 5-Hydroxypropafenone from Individual Human Serum Proteins. 133

4.4. Binding Characteristics Propafenone and 5-Hydroxypropafenone to Isolated Proteins 135
4.4.1 Binding Characteristics of Propafenone and 5-Hydroxypropafenone in a Solution of AAG. 135
4.4.2 The Binding Capacities Propafenone and 5-Hydroxypropafenone in a Solution of Albumin. 138
4.4.3 Characterization of Propafenone and 5-Hydroxypropafenone Binding in Serum. 138
4.4.4 Binding Characteristics of Propafenone and 5-Hydroxypropafenone to Lipoproteins. 140
4.4.5 CALCULATED BINDING OF PROPAFENONE AND 5-HYDROXYPROPAFENONE BY MATHEMATICAL RECONSTITUTION OF THE DRUG-BINDING SYSTEM.

4.5 UPTAKE OF PROPAFENONE AND 5-HYDROXYPROPAFENONE BY HUMAN RED BLOOD CELLS.

4.6 SUMMARY

5. CONCLUSION

6. REFERENCES

7. APPENDICES
# List of Tables

1. Comparison of non-specific binding of 5-hydroxypropafenone and propafenone to the acrylic equilibrium dialysis apparatus following incubation for 8 hours at 37°C and to the MPS-1 ultrafiltration devices at 25°C.  
   PAGE 60

2. Change in total protein and alpha-1-acid glycoprotein in plexiglass dialysis cells and cellulose dialysis membranes during dialysis.  
   PAGE 70

3. Binding parameters of 5-hydroxypropafenone in serum of healthy humans  
   PAGE 71

4. 5-hydroxypropafenone free fraction in normal sera with increasing concentrations of 5-hydroxypropafenone  
   PAGE 75

5. Assessment of the binding of the metabolite 5-hydroxypropafenone in vitro by equilibrium dialysis and ultrafiltration with and without the addition of therapeutic concentrations of the parent compound propafenone.  
   PAGE 76

6. Displacement of propafenone and 5-hydroxypropafenone from human serum protein binding sites by disopyramide and ibuprofen  
   PAGE 84

7. The composition of different classes of lipoproteins [VLDL, LDL, and HDL]  
   PAGE 141
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Structure of propafenone and the active metabolites 5-hydroxypropafenone and n-depropyl-propafenone</td>
</tr>
<tr>
<td>2.</td>
<td>Time required to attain equilibrium for the equilibrium dialysis of 5-hydroxypropafenone.</td>
</tr>
<tr>
<td>3.</td>
<td>The pH dependent binding of propafenone (2.0 μg/mL) in serum, and solutions of HSA and AAG.</td>
</tr>
<tr>
<td>4.</td>
<td>The pH dependent binding of 5-hydroxypropafenone (0.5 μg/mL) in serum, and solutions of albumin and AAG.</td>
</tr>
<tr>
<td>5.</td>
<td>Influence of alterations in pH on the partitioning of propafenone (2.0 μg/mL) between aqueous and organic layers in a toluene phosphate buffer system.</td>
</tr>
<tr>
<td>6.</td>
<td>Influence of alterations in pH on the partitioning of 5-hydroxypropafenone (0.5 μg/mL) between aqueous and organic layers in various organic solvents.</td>
</tr>
<tr>
<td>7.</td>
<td>The effect of molar strength of phosphate buffer on the free fraction of 5-hydroxypropafenone in serum ± SD.</td>
</tr>
<tr>
<td>8.</td>
<td>The relationship between the ratio of bound drug concentration/free drug concentration ± SD vs. bound drug concentration of 5-hydroxypropafenone in serum.</td>
</tr>
<tr>
<td>10.</td>
<td>Cont.</td>
</tr>
<tr>
<td>10.</td>
<td>Comparison of binding of propafenone (2.0 μg/mL) and 5-hydroxypropafenone (0.5 μg/mL) in AAG, HSA, HSA + AAG, serum and lipoprotein deficient serum.</td>
</tr>
<tr>
<td>11.</td>
<td>The effect of buffer vs. human serum ultrafiltrate as solvents to dissolve AAG, HSA, and AAG + HSA on the free fraction (± SD) of propafenone (2.0 μg/mL).</td>
</tr>
<tr>
<td>12.</td>
<td>The effect of buffer vs. human serum ultrafiltrate as solvents to dissolve AAG, HSA, and AAG + HSA on the free fraction (± SD) of 5-hydroxy-propafenone (0.5 μg/mL).</td>
</tr>
</tbody>
</table>
12. Comparison between the free fraction (± SD) of propafenone (2.0 µg/mL) + 5-hydroxypropafenone (0.5 µg/mL) in free fatty acid free albumin, and in HSA. 82

13. Comparison of the free fraction (± SD) of propafenone (2.0 µg/mL) and 5-hydroxypropafenone (0.5 µg/mL) in lipoprotein deficient serum, and normal serum. 83

14. The effect of the addition of ibuprofen (50.0 µg/mL) on the free fraction (± SD) of propafenone (2.0 µg/mL) and 5-hydroxypropafenone (0.5 µg/mL). 86

15. The effect of the addition of disopyramide (8.0 µg/mL) on the free fraction (± SD) of propafenone (2.0 µg/mL) and 5-hydroxypropafenone (0.5 µg/mL). 87

16. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in a physiological concentration of AAG (19.3 µM) in 0.1 phosphate pH 7.40 buffer. 89

17. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in a physiological concentration of HSA (630.0 µM) in 0.1 phosphate pH 7.36 buffer. 91

18. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in serum. 95

19. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in a physiological concentration of HDL (15.3 µM complex, 1.72 mg/mL total protein) in 0.1 M phosphate buffer pH 7.32. 96

20. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of PF and 5-hydroxypropafenone in a physiological concentration of LDL (1.8 µM complex, 0.84 mg/mL total protein) in 0.1 M phosphate buffer pH 7.32. 98

21. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in a physiological concentration of VLDL (0.17 µM complex, 0.09 mg/mL total protein) in 0.1 M phosphate buffer pH 7.32. 100
22. Free fraction ± SD of propafenone at various concentrations in solutions of human serum proteins.

23. Free fraction ± SD of 5-hydroxypropafenone at various concentrations in solutions of human serum proteins.

24. A direct plot of propafenone binding (bound vs. free) calculated by mathematically reconstructing binding contributions of serum protein constituents and subsequent removal of binding contributions of individual serum proteins.

25. A direct plot of 5-hydroxypropafenone binding (bound vs. free) calculated by mathematically reconstructing binding contributions of serum protein constituents and subsequent removal of binding contributions of individual serum proteins.

26. Comparison of the ratio (drug concentration in RBC/drug concentration in supernatant) of propafenone and 5-hydroxypropafenone with buffer, serum, and plasma as supernatants.

27. Theoretical Rosenthal plot of drug-protein binding fitting a binding model for a system containing one drug binding specific site + non-specific binding site. The binding capacity of the high affinity site is 1.0 and the non-specific binding (Kns) is held constant at 2; only the affinity of the high affinity site was altered.

28. Theoretical plot of free fraction of drug-protein binding fitting a model containing one specific site + non-specific binding. Binding constants (i.e. the capacity of the high affinity site is 1.0, and non-specific binding is 2) are held constant.
TABLE OF APPENDICES

1. PROTEIN COMPOSITION OF NORMAL VOLUNTEERS FOR BINDING STUDY  161
2. PROTEIN CONTENT (TOTAL PROTEIN AND $\alpha$-1-ACID GLYCOPROTEIN) IN SOLUTIONS ISOLATED PROTEINS USED IN BINDING EXPERIMENTS.  162
3. COMPOSITION OF HUMAN SERUM ULTRAFILTRATE  163
4. ELECTROPHORESIS RESULTS FOR ISOLATED PROTEINS USED IN BINDING EXPERIMENTS. LANE 1 STANDARD PROTEIN SOLUTIONS, LANE 2 HUMAN SERUM ULTRAFILTRATE, LANE 3 AAG (UNDILUTED), LANE 4 HSA, LANE 5 HSA + AAG, LANE 6 NORMAL CONTROL SERUM, LANE 7 LIPOPROTEIN DEFICIENT SERUM  164
5. PROTEIN CONCENTRATIONS IN SOLUTIONS OF ISOLATED HUMAN SERUM PROTEINS (AAG, HSA, HDL, LDL, AND VLDL) USED IN EXPERIMENTS IN WHICH BINDING WAS CHARACTERIZED  165
6. AGAROSE ELECTROPHORESIS OF LIPOPROTEINS BEFORE AND AFTER EQUILIBRIUM DIALYSIS FOR 8 HOURS AT 37 °C.  166
**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.V.</td>
<td>atrioventricular</td>
</tr>
<tr>
<td>AAG</td>
<td>α-1-acid glycoprotein</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>ANOVA</td>
<td>one way analysis of variance</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>D</td>
<td>free drug (unbound)</td>
</tr>
<tr>
<td>DP</td>
<td>drug-protein complex</td>
</tr>
<tr>
<td>ECD</td>
<td>electron capture detection</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>extensive metabolizers</td>
</tr>
<tr>
<td>FF</td>
<td>free fraction</td>
</tr>
<tr>
<td>GLC</td>
<td>gas-liquid chromatography</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HP</td>
<td>Hewlett Packard</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>I.S.</td>
<td>internal standard</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
</tr>
<tr>
<td>Ka</td>
<td>binding association constant</td>
</tr>
<tr>
<td>Kns</td>
<td>binding constant for non-specific binding to protein</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>M.W.</td>
<td>molecular weight</td>
</tr>
<tr>
<td>n</td>
<td>number of homogeneous binding sites</td>
</tr>
<tr>
<td>NP</td>
<td>capacity of binding protein</td>
</tr>
<tr>
<td>P</td>
<td>free protein (unbound)</td>
</tr>
<tr>
<td>PF</td>
<td>propafenone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PM</td>
<td>poor metabolizers</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafluoeethylene</td>
</tr>
<tr>
<td>r</td>
<td>molar ratio of drug bound over total protein</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RID</td>
<td>radial immunodiffusion</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide electrophoresis</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>$T_{1/2}$</td>
<td>elimination half-life</td>
</tr>
<tr>
<td>$V_d$</td>
<td>volume of distribution</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
</tr>
</tbody>
</table>
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This thesis is dedicated to

my loving and supportive family.

P.S. Dad if you could only see me now!
1. INTRODUCTION

1.1 PROPafenONE

1.1.1 PHARMACOLOGY OF PROPafenONE

Propafenone (PF) is a potent class 1C [Vaughan-Williams] antiarrhythmic agent (Figure 1). In several in vitro studies PF was found to act primarily on the fast sodium channels, causing a decrease in the rate of the rise of phase 0 of the action potential [Dukes and Vaughan-Williams, 1984]. In addition, PF displays mild beta-blocking effects (1/8 potency of propranolol in guinea-pig cardiac tissue) [Harron and Brogden, 1987], prolongs action potential duration [Dukes and Vaughan-Williams, 1984], and has very mild calcium channel blocking effects (1/100 potency of verapamil in guinea-pig ventricular strips) [Harron and Brogden, 1987]. Therapeutic concentrations in humans showed PF to have the same activity as PF in vitro [Connolly et al., 1983; Muller-Peltzer, 1983]. These actions increase the PR, QT, and HV intervals, indicating a slowing of the action potential transmission through the heart. PF was also found to increase effective and functional refractory periods, and to increase A-V nodal conduction time [Connolly et al., 1983; Muller-Peltzer, 1983; Kerr et al., 1988].

Propafenone is marketed as a racemate, that is, consisting of an equal amount of (+)-S-propafenone and (-)-R-propafenone randomly mixed. It has been shown that both enantiomers produce similar
frequency-dependent depression of maximum upstroke of phase 0 in canine Purkinje fibers [Kroemer et al., 1989]. In addition, the (+)-S-PF enantiomer was shown to have a 100 fold greater affinity for human lymphocyte $\beta_2$-receptor as compared to the (-)-R-PF enantiomer [Kroemer et al., 1989].

1.1.2 THERAPEUTIC EFFICACY OF PROPAFENONE

PF has been found to be effective in the treatment of a number of different arrhythmias, including recurrent atrial fibrillation [Kerr et al., 1988; Ludmer, 1987], atrial tachyarrhythmias after cardiac surgery [Connolly et al., 1987], Wolff-Parkinson-White Syndrome [Ludmer, 1987], paroxysmal supraventricular arrhythmias [Hammill et al., 1988], ventricular premature complexes [Rabkin et al., 1987], and chronic ventricular arrhythmias [Schlepper, 1987].

As with other antiarrhythmic agents, PF has adverse effects; however, when compared to other class 1C antiarrhythmic agents, these tend to be less common. The most serious of these adverse effects are the cardiovascular; arrhythmogenesis, aggravation of arrhythmias, decreased left ventricular function (negative inotropic effects), and conduction blocks [Schlepper, 1987]. Side effects of a less serious nature are nausea, GI distress, change in taste perception, dizziness, paresthesias, and visual blurring [Schlepper, 1987]. Approximately three percent of patients with adverse effects require discontinuation of therapy [Harron and Brogden, 1987].
1.1.3 PHARMACOKINETICS OF PROPafenONE

1.1.3.1 ABSORPTION OF PROPafenONE

Absorption of PF in normal subjects is almost complete. In a study using deuterated PF, 95% of the oral dose was absorbed, with only 1% of the dose excreted unchanged in the faeces [Hege et al., 1984]. Oral bioavailability of PF appears to be dose-dependent [Hollmann et al., 1983]. For example, bioavailability for 150mg, 300mg, and 450mg oral doses of PF was 13%, 32%, and 55% respectively, indicating possible saturable first-pass metabolism [Hollmann et al., 1983]. In addition, it was also found that food substantially increased (mean value 147%) the bioavailability in extensive metabolizers [Axelson et al., 1987].

1.1.3.2 DISTRIBUTION AND PLASMA PROTEIN BINDING OF PROPafenONE

The volume of distribution for PF is estimated to be between 2-4.5 L/kg of body mass [Schlepper, 1987]. PF is also extensively plasma protein bound (~ 95-97%) at therapeutic concentrations [Chan et al., 1989b]. Two binding sites for PF have been identified in whole serum, one low-capacity high-affinity site thought to be associated with alpha-1-acid glycoprotein (AAG), and one high-capacity low-affinity binding site believed to be associated with serum albumin [Chan et al., 1989b]. PF also undergoes concentration-dependent plasma protein binding in vitro, at levels well above the therapeutic concentration [Chan et al. 1989b]. PF and its major metabolite 5-
hydroxypropafenone (5-OH-PF) were found to distribute primarily to liver, lung, and heart [Latini et al., 1987].

1.1.3.3 METABOLISM OF PROPAFENONE

PF metabolism exhibits genetic polymorphism, in that subpopulations of (-90%) extensive metabolizers (EM) and (-10%) poor metabolizers (PM) exist for the drug [Harron and Brogden, 1987]. PMs were characterized by high concentrations of PF, existence of a linear dose-response relationship, long $T_\frac{1}{2}$ (17.2 ± 8.0 h), low oral clearance (264 ± 48 mL/min), and very little accumulation of 5-hydroxypropafenone (5-OH-PF) [Siddoway et al., 1987]. EMs, on the other hand, were characterized by lower PF levels at steady-state, no linear dose-response relationship, short $T_\frac{1}{2}$ (5.5 ± 2.1 h), high oral clearance (1115 ± 1238 mL/min), and detectable levels of 5-OH-PF [Siddoway et al., 1987]. EMs showed the greatest increase in bioavailability with the consumption of food, while a PM showed virtually no increase in bioavailability due to food [Axelson et al., 1987]. As well, with chronic dosing of PF, the ratio of 5-OH-PF/PF decreases from the single dose situation. This may be due to the saturation of the metabolic process [Giani et al., 1988].

Propafenone also undergoes stereoselective disposition in patients on long term therapy [Kroemer et al., 1989]. It has been demonstrated that the ratio of area under the curve for (+)-S-PF to (-)-R-PF was 1.73 ± 0.15 [Kroemer et al., 1989].
PF is extensively metabolized, primarily to 5-OH-PF, 4-methoxy-5-OH-PF, and N-depropyl-PF, followed by conjugation and excretion of the respective glucuronide or sulfate. The glucuronide and sulfate of 5-OH-PF are, by far, the most common metabolites of PF, followed in quantitative importance by the conjugates of 4-methoxy-5-hydroxy-PF, and PF itself. The major route of excretion of metabolites (conjugated and non-conjugated) in humans is via the faeces (-53%) [Hege et al., 1984]. Urinary excretion of conjugated and unconjugated drug and metabolites accounts for approximately 18% of the total dose [Hege et al., 1984]. The most prominent metabolite in the urine is 5-OH-PF [Hege et al., 1984].

It has also been observed that the metabolites 5-OH-PF and N-depropyl-PF accumulate during chronic dosing in patients in which arrhythmias were the only disease state reported [Kates et al., 1985]. Plasma levels of 5-OH-PF and N-depropyl-PF accumulate to 23% and 18% of the total steady-state plasma level of PF, respectively [Kates et al., 1985] (Figure 1).

Figure 1. Structure of propafenone and the active metabolites 5-hydroxypropafenone and n-depropylpropafenone.
The metabolite, 5-OH-PF, also has much the same activity as the parent drug in vitro. In vitro studies indicate that 5-OH-PF has a somewhat greater negative inotropic and calcium channel blocking activity than PF [Dukes and Vaughan-Williams, 1984]. However, in whole animal studies 5-OH-PF was found to be twice as efficacious as PF. This may be attributed to the smaller volume of distribution \(V_d\) for 5-OH-PF. N-depropyl-PF is also active, but, its activity has been less well defined [Malfatto et al., 1988]. Similar results for 5-OH-PF have been reported in canine Purkinje fibers [Thompson et al., 1987] and in guinea pig ventricular muscle [Rouet et al., 1989].

To date there have been no accurate correlations between drug concentration and observed pharmacological response of propafenone. It has been shown that the effective dosage is not significantly different between EMs and PMs, even though the concentration of PF is substantially higher in PMs [Siddoway et al., 1987]. It has been shown that propafenone has two active metabolites which accumulate upon chronic dosing, namely 5-OH-PF and n-depropyl-PF [Kates et al., 1985]. Both of these metabolites have been shown to possess similar activity to that of propafenone [Malfatto et al., 1988]. Further, it was shown that when the concentrations of PF, 5-OH-PF, and AAG were used to predict the QRS width, a better correlation was observed than when only the concentration of PF was used [Chan, 1989c]. It appears that both the concentration of propafenone and 5-hydroxypropafenone, in addition to the drug-protein binding, must be considered to arrive at a reasonable estimate for the dose-response relationship for
propafenone in EMs. However, to date there is no estimation of 5-OH-PF binding with the addition of the parent drug PF, and thus the free concentration of 5-OH-PF.

1.2 DRUG-PROTEIN BINDING

The binding of drugs to plasma and tissue proteins can have a large influence on both the pharmacokinetics and pharmacodynamics, and therefore, a drug's therapeutic effect, or toxicity [Wilkinson, 1983]. If drug-protein binding exceeds 90%, alterations in binding can have large effects on the variability of the estimated pharmacokinetic parameters of drugs (i.e. a 10% decrease in the binding will increase the free drug concentration by approximately 100%) [Svensson et al., 1986]. For example, since the extent of binding is directly correlated to the hepatic clearance of a low clearance drug, alterations in binding will directly affect the clearance of total drug, and therefore, the total drug concentration. Once equilibrium has been attained, the free drug concentration of a low clearance drug will be similar to that observed before the changes in binding. On the other hand, since the clearance of a high clearance drug is independent of the free drug concentration, altered binding may not significantly alter the total drug concentration but can increase or decrease the free drug concentration [Svensson et al., 1986].

It is generally accepted that the free drug concentration is responsible for the majority of the observed pharmacological response, since it is free to distribute to the site of action. Since the free
drug concentration of a high clearance drug may be changed due to alterations in binding, the observed pharmacodynamics of this drug may also be altered [Svensson et al., 1986]. These changes in free drug would be particularly important for drugs with a steep dose-response relationship, such as lidocaine [Harrison and Alderman, 1971]. Furthermore, depending on the chemical nature of the drug (lipophilic or hydrophilic), binding can also influence pharmacokinetic parameters such as the volume of distribution [Lindup, 1987; Wilkinson, 1983].

Thus, alterations in binding can directly influence primary pharmacokinetic parameters such as clearance and distribution [Lindup, 1987]. Since alterations in these pharmacokinetic parameters may have large effects on the free drug concentration, and therefore, observed therapeutic effect or toxicity, it is important to characterize the drug-protein binding interactions in order to be able to predict binding alterations due to the presence of other drugs, and/or disease.

Many techniques, such as circular dichroism [Gamel-El et al., 1982; Chignell, 1969], fluorescent probes [Essassi et al., 1989], calorimetric studies [Akiand and Yamamoto, 1989], gel filtration, and ultracentrifugation [Kwong, 1985] are available to evaluate drug-protein interactions. Techniques such as, circular dichroism, fluorescent probes, and calorimetric studies are generally used to determine the nature of the drug-protein interaction, whereas gel filtration, ultracentrifugation, ultrafiltration, and equilibrium dialysis provide more quantitative information of the drug-protein
binding interaction (e.g. free fraction of drug in serum). Despite the large number of available methods to study the drug-protein interaction, the most commonly used methods are ultrafiltration and equilibrium dialysis [Lindup, 1987; Kwong, 1985]. Both equilibrium dialysis and ultrafiltration must be rigorously validated, and carefully controlled to provide accurate and reproducible estimates of drug-protein binding [Lindup, 1987; Kwong, 1985].

1.2.1 ULTRAFILTRATION

The method of ultrafiltration relies on a pressure gradient, usually due to centrifugation, which forces plasma water and dissolved solutes (drugs, ions, and small endogenous compounds) through a semipermeable membrane. The membrane is assumed to be "ideal", in that it should not discriminate between drug and water molecules [Kwong, 1985]. One of the drawbacks to using ultrafiltration, particularly if the molecular weight of the ligand of interest is greater than 300, is molecular sieving [Kwong, 1985]. This occurs as a result of proteins packed on to the membrane due to centrifugation which discriminates between water molecules and ligand, such that water molecules pass through the membrane unobstructed in comparison to drug molecules [Kwong, 1985]. Ultrafiltration is a fast and easy method for the determination of drug-protein binding provided that, in addition to molecular sieving, factors such as pH, temperature, and non-specific binding are accurately accounted for, or controlled [Lindup, 1987; Kwong, 1985].
1.2.2 EQUILIBRIUM DIALYSIS

Equilibrium dialysis is considered the "classical" method for the determination of drug-protein binding [Lindup, 1987]. Equilibrium dialysis works on the principle that only free or unbound drug in serum will equilibrate across a semi-permeable membrane. Thus, once equilibrium has been attained, drug concentration in the buffer equals the free drug concentration in the serum compartment. If aliquots of serum and buffer are measured and binding calculated, non-specific binding does not contribute to the calculated binding [Kwong, 1985]. As with ultrafiltration, equilibrium dialysis must be carefully optimized and controlled [Kwong, 1985; Lindup, 1987].

Initially, time to equilibrium must be established. Time to equilibrium is directly affected by the pore size of the membrane, the surface (membrane) to volume ratio [Lindup, 1987], and the side to which the drug is added [McNamarra and Bogardu, 1982]. Theoretically, if drug is added to the serum compartment, equilibrium will be obtained earlier. Equilibrium can usually be established within 4-18 hours for most drugs [Kwong, 1985; Tozer et al., 1981].

Along with longer dialysis times comes unique time dependent problems, such as, volume shifts, and pH shifts. Volume shifts occur as a result of unequal osmotic and colloidal pressure between the buffer and serum compartment of the dialysis cell [Kwong, 1985; Lindup, 1987]. Serum contains many ions and endogenous compounds, in addition to large macromolecules, which contribute to osmotic.
pressure. The addition of a buffer of a lower osmotic pressure to the other side of the dialysis membrane can result in an osmotic pressure gradient. This gradient may pull water from the buffer compartment into the serum, thus diluting serum proteins, and resulting in lower calculated binding [Kwong, 1985]. Volume shifts can be directly measured by comparing volumes pre- and post-dialysis, or by measuring a non-diffusible marker, such as serum protein concentration, pre- and post-dialysis. Generally small volume shifts start to appear after 6-8 hours of dialysis [Lima et al., 1981].

A long dialysis time (24 hours) may significantly increase the pH in the dialysis system, due to the loss of dissolved carbon dioxide in the serum compartment [Lui and Chiou, 1986]. Since many drugs are known to undergo pH dependent binding the buffer chosen must have a large enough capacity to oppose this shift [Lindup, 1987; Kwong, 1985]. However, buffer strength and composition have been shown to affect the binding of a number of drugs, such as propranolol and theophylline [Kwong, 1985]. Discussion has focused on the use of physiological buffers; however, their use has been shown not to offer much improvement to the presently used "non-physiological" phosphate buffers, since artificial intervention is usually required to control pH and adjust pH prior to dialysis [Kristensen and Grams, 1982].

Donnan effects, which are the result of an unequal distribution of charged compounds between the buffer and serum compartment of the dialysis cell have also been shown to alter drug-protein binding [Kwong, 1985; Lindup, 1987]. Since proteins at physiological pH are
charged, and usually confined by the semipermeable membrane, unequal diffusion of charged particles can lead to alterations in binding. This is particularly true for compounds which are weakly bound, and strongly ionized in the dialysis system [Kwong, 1985]. This can usually be corrected by the addition of diffusible ions such as sodium and chloride to the buffer in order to electrostatically equilibrate the dialysis system [Kwong, 1985].

Protein leakage as a result of membrane rupture, and/or improper mounting of the dialysis membrane must also be considered. The presence of impurities, particularly in radiochemical studies, must be ruled out since the possible displacement of the drug of interest by an impurity may result in erroneous drug-protein binding data [Kristensen and Gram, 1982].

When these factors are optimized and controlled, equilibrium dialysis can provide accurate, and some suggest, more precise measurements of binding compared to other methods, such as ultrafiltration [Kurtz et al., 1977; Kwong, 1985]

1.3 SERUM PROTEINS

Several proteins, normal constituents of serum, can bind both endogenous, and exogenous compounds. These proteins are, human serum albumin (HSA), α-1-acid glycoprotein (AAG), lipoproteins, pre-albumin, δ-globulins, α-1-antitrypsin, and various other specific transport proteins [Routledge, 1986; Kremer et al., 1988; Kragh-Hansen, 1981].
However, for most drug compounds examined, HSA, AAG and lipoproteins account for the majority of observed binding [Routledge, 1986].

1.3.1 HUMAN SERUM ALBUMIN

Human serum albumin (HSA) (molecular weight 69000 Daltons), accounts for approximately 60% of the total plasma protein concentration [Svensson et al., 1986]. HSA is thought to be important in the storage and transport of various endogenous ligands in serum (free fatty acids, chloride ions, bilirubin, steroids, and tryptophan), transport amino acids to tissues [Kragh-Hansen, 1981], and to provide colloid osmotic pressure in the blood.

Over 20 genetic variants of HSA have been identified in the human population; however, these variants are rare in the European population. It is not clear if these variants cause altered binding of endogenous and exogenous ligands [Kragh-Hansen, 1981].

HSA is considered to be the most important drug-binding protein in plasma due to its large concentration (approximately 600 μM)[Routledge, 1986]. In addition, albumin may also play a role in the tissue binding of drugs, since approximately 60% of albumin is contained in extravascular tissue [Routledge, 1986]. Six structurally specific binding sites have been identified on the HSA molecule. HSA is known to bind acidic, basic and neutral compounds, but is generally considered to bind mainly anionic (acidic) ligands with high affinity [Kragh-Hansen, 1981]. It has been shown that the conformation of HSA
can change due to changes in pH, and upon binding of certain specifically bound ligands [Kragh-Hansen, 1981].

1.3.2 α-1-ACID GLYCOPROTEIN

α-1-acid glycoprotein (AAG) is one of the 90 other globulin proteins present in serum [Routledge, 1986]. The exact physiological role of AAG is not well understood; however, it has been shown to increase up to 2-3 fold in traumatic injuries [Routledge, 1986; Kremer et al., 1988]. AAG has also been shown to be elevated in certain chronic diseases including cancer, rheumatoid arthritis, chronic renal failure and Crohn’s disease [Kremer et al., 1988]. This has led to speculation that AAG is a mediator of normal coagulation, immunological and tissue repair processes [Kremer et al., 1988].

AAG appears to be an important binding protein of basic and neutral drugs both in serum and in tissue, since approximately 40% of the protein is in tissue [Routledge, 1986]. Approximately 40% of the AAG molecule is carbohydrate, some of which is composed of sialic acid, which results in the AAG molecule being acidic with a low pKa in serum [Kremer et al., 1988]. Despite the acidic nature of the protein, the majority of drug-protein binding interactions appear to be hydrophobic in nature [Muller, 1989; Routledge, 1986; Kremer et al., 1988].

It is not yet clear as to the number of binding sites present on the AAG molecule; however one high affinity, low capacity site for
basic and neutral drugs has been identified (binding affinity ($K_a$) $\approx 10^4 - 10^7$ M$^{-1}$ and capacity $\approx 10^{-5}$ M) [Routledge, 1986]. Thus, fluctuations in AAG concentration can have a large affect on the degree of drug-protein binding of most basic drugs. Acidic drugs, such as warfarin, have also been shown to bind to AAG and compete for binding sites with basic drugs [Otagiri et al., 1987].

1.3.3 SERUM LIPOPROTEINS

Lipoprotein complexes are mixtures of protein, phospholipids, cholesterol and triglycerides. The function of these complexes is the transport of lipids within the body. High, low and very low density lipoproteins make up the complex transport system within the blood stream, whereas chylomicrons are largely responsible for the transport of digested fats from the intestinal mucosa to the blood stream [Ganong, 1985]. Very low density lipoproteins (VLDL) are formed in the liver and serve to transport triglycerides from the liver to extrahepatic tissue. Thus, VLDLs are composed of low concentrations of protein, phospholipid, cholesterol, and very high concentrations of triglycerides. Once the triglycerides are largely removed, the VLDL becomes an intermediate density lipoprotein (IDL). The IDL picks up excess cholesterol from the high density lipoproteins (HDL), and then becomes the low density lipoprotein (LDL). The purpose of the LDL is to provide tissue with cholesterol; therefore, the composition of the LDL has a higher concentration of cholesterol in comparison to the other lipoproteins examined [Ganong, 1985]. Finally, the HDL provides a means for removing excess cholesterol from cells (the concentration
of proteins in much higher in the HDL in comparison to the other lipoproteins) [Ganong, 1985]. Lipoproteins can be elevated in various types of diseases, such as familial hyperlipidemias. For example in familial hypercholesterolemia heterozygous form D, the concentration of LDL may be significantly elevated [Brown and Goldstein, 1985].

It has been observed that both lipophilic and cationic drugs tend to bind, or are solubilized within the lipoprotein complex [Routledge, 1986]. It should be noted that not much is known about the actual drug-protein/lipid interaction for lipoproteins.

1.3.4 UPTAKE OF DRUGS INTO RED BLOOD CELLS

The uptake of drug by the red blood cell may contribute to the distribution of a drug, and therefore, influence the pharmacokinetic parameters dependent on distribution. It is generally considered that drug intrinsic clearance expressed in terms of plasma, is approximately the same as blood intrinsic clearance; however, when drug uptake by red blood cells is greater than that observed in plasma, a large overestimation in the plasma intrinsic clearance will occur [Gibaldi and Perrier, 1982]. The accurate estimation of drug present in the red blood cell can have implications on the pharmacokinetic parameters calculated, and therefore, must be investigated.
1.3.5 CHEMICAL BASIS FOR DRUG-PROTEIN INTERACTION

As identified above, a number of different chemical interactions can account for the drug-protein interaction (ionic bonding, hydrogen bonding, Van der Waals forces, and hydrophobic interactions) [Lindup, 1987]. The degree to which each binding force contributes to the overall drug-protein interaction is dependent on both the drug and the protein involved in this interaction. In fact, some chemical forces may even reduce the strength of the drug-protein interaction (e.g. ionic repulsion).

HSA, for example, tends to undergo a number of various chemical interactions with ligands, since there are six well defined specific binding sites on HSA. The diazepam binding site tends to favor ligands with an aromatic carboxyl group and an elongated hydrophobic group. The arylpropionates, such as ibuprofen, fenoprofen, and naproxen, tend to favor binding to this site on HSA. This would suggest that both ionic and hydrophobic interactions are important in the binding of these drugs to this particular HSA binding site [Kragh-Hansen, 1981].

The binding of basic drugs to the AAG molecule tends to be determined largely by specific hydrophobic interactions. It was shown that the desialation of AAG does not affect the binding of propranolol [Kremer et al., 1988; Muller, 1989]. This would suggest that the sialic acid residues on the protein do not contribute to the binding of propranolol.
The binding interaction with lipoproteins may be largely dictated by a solubilization process. It should however be pointed out that the binding of quinidine was found to be saturable, indicating a possible "classical" binding interaction [Nilsen, 1976].

The conformation and configuration of a drug may also determine the strength of the binding interaction. Both AAG and HSA have shown stereoselective drug-protein interactions. This indicates that very specific binding regions, which can discriminate between the configuration of drugs, are present on AAG and HSA [Kremer et al., 1988; Kragh-Hansen, 1981].

1.4 QUANTITATIVE DESCRIPTION OF THE DRUG-PROTEIN BINDING SITE

Although it is important to determine the chemical nature of the drug-protein interaction, it is also important to determine the quantitative nature of the drug-protein interaction. Quantitative drug-binding, in addition to providing information regarding the free drug concentration, also serves as a predictive tool. Factors including, at what concentration binding to a protein becomes non-linear, the changes in binding due to altered protein concentrations, and the possible occurrence of binding displacements due to the addition of a second drug may be predicted with quantitative drug-protein data [Svensson et al., 1986]. The binding capacity and affinity (strength of the drug-protein interaction) can be derived from first principles. Any reversible drug-protein binding
interaction in serum or tissue can be described by equation 1.

\[ [D] + [P] \rightleftharpoons \frac{K_1}{K_2} [DP] \]  

(1)

where D and P are the unbound drug and protein, respectively, and DP is the drug protein complex. The constants for the forward and backward reactions are K1 and K2, respectively. Following the law of mass action, at equilibrium the apparent association constant (Ka), the strength of the binding interaction, can be given by equation 2.

\[ Ka = \frac{K_1}{K_2} = \frac{[DP]}{[D][P]} \]  

(2)

From equation 2 it can be shown that the concentration of bound drug can be given by the product of the affinity constant and the concentrations of free drug and protein ([DP] = Ka[D][P]). Binding can be represented as the molar ratio of drug bound (r) (Equation 3)

\[ r = \frac{[DP]}{[DP] + [P]} \]  

(3)

This ratio is the concentration of bound drug or protein over the total protein concentration (Pt) [Pt] = [DP] + [P]. Substituting for [DP], since [DP] cannot be measured directly, equation 4 is derived.

\[ r = \frac{Ka[D][P]}{Ka[D][P] + [P]} = \frac{Ka[D]}{1 + Ka[D]} \]  

(4)

This can be simplified to show the bound concentration [DP] as in
equation 5

\[ [\text{DP}] = \frac{K_a [\text{Pt}][\text{D}]}{1 + K_a [\text{D}]} \]  

and for (i) number of different binding sites (equation 6).

\[ [\text{DP}] = \sum_{j=1}^{i} \frac{(n_j [\text{Pt}][\text{D}] K_a)}{1 + K_a [\text{D}]} \]  

In equation 5 and 6 the capacity of the binding site is the number of binding sites per protein molecule (n) multiplied by the actual concentration of that protein.

Due to the non-linear nature of the equations representing drug-protein binding (equation 5), it has become difficult to graphically determine both the capacity and the affinity of the binding sites. Several transformations of the binding data have been presented to overcome this problem. The Klotz reciprocal plot (equation 7)

\[ \frac{1}{r} = \frac{1}{n[D]K_a} + \frac{1}{n} \]  

is analogous to the Lineweaver-Burke plot or the Langmuir adsorption isotherm. The problem encountered with this transformation is that it places more emphasis on the smaller values of r which potentially have the greatest amount of experimental error [Svensson et al., 1986; Klotz, 1983].
The most widely used transformation of binding data has been the Scatchard transformation [Scatchard, 1949](Equation 8).

\[ r = \frac{nK_a - rK_a}{[D]} \]  

(8)

The use of this transformation has been widespread, and in many cases, neglecting the assumptions made during the derivation of the Scatchard plot, that:

1. The drug-protein interaction is reversible
2. All binding sites behave as if they have the same affinity.
3. Data must be obtained at equilibrium
4. Binding of one molecule of ligand does not negatively or positively affect the binding of subsequent molecules of ligand.
5. No binding artifacts are present.

The third transformation is the Rosenthal plot presented in equation 9.

\[ \frac{[DP]}{[D]} = n[P_t]K_a - [DP]K_a \]  

(9)

When more than one different binding site is present for a ligand, these transformations lose their linearity, and any potential benefit in graphically determining the binding parameters is lost [Klotz, 1982; Klotz, 1983; Burgisser, 1984]. This has led to the estimation of binding parameters (capacity and affinity) by non-linear
fitting of either Scatchard and Rosenthal equations. The use of Scatchard and Rosenthal analysis, has shown to be incorrect from the statistical standpoint, since the y observation is not independent of the x observation [Svensson et al., 1986]. That is, rather than having y as the function of x (y = f(x)) the Rosenthal and Scatchard analysis have y as a function of both x and y (y = f(y/x)).

An alternative method is non-linear curve fitting of the original binding isotherm (equation 5 and 6). Using programs such as LIGAND\textsuperscript{R}, ENZFITTER\textsuperscript{R}, and NONLIN\textsuperscript{R}, equations 5 and 6 can be entered and the data fit to the most appropriate equation. This truly analyzes the dependent vs. the independent parameters to arrive at the estimation of the binding parameters [Burgisser, 1984; Klotz, 1982; Klotz, 1983].

1.5 RATIONALE

Like many other antiarrhythmics, PF appears to display a steep dose-response relationship [Connolly et al., 1983b; Siddoway et al., 1984]. This suggests that small changes in PF concentration may result in disproportionate changes in effect, which could lead to toxicity or lack of pharmacological effect. Therefore, factors which directly affect PF concentration, such as the elimination and disposition of PF, must be documented and carefully examined.

Propafenone is highly bound (~96% within the therapeutic range of 0.5-2.0 ug/mL) in serum of normal healthy volunteers [Chan et al.,
1989b]. Moreover, it has been shown that PF binding in the serum is largely dependent on the concentration of α-1-acid glycoprotein (AAG) [Chan et al., 1989b; Gilles et al., 1985], which may increase 2-3 fold in response to trauma and various disease states [Routledge, 1986]. Since, PF is considered a flow-dependent drug, changes in protein binding of PF (i.e. displacement and altered concentrations of AAG) would not be expected to alter the pharmacokinetic parameters of PF unless significant changes in distribution occurred in response to these changes in free fraction. Although free drug concentration may increase in response to displacement, or decrease as a result of increasing AAG concentration, the total plasma drug concentration may appear unchanged. This is important considering the generally accepted pharmacological premise that free drug is usually responsible for pharmacological activity. Therefore, it seems important to establish not only those factors which elicit changes in total plasma PF concentrations, but also changes in plasma free drug concentration of PF. Information regarding factors controlling the concentration of free (pharmacologically active) drug in the plasma would contribute substantially to the understanding of the as yet, undefined plasma concentration vs. effect relationship for PF.

Experiments using perfused rabbit hearts have further demonstrated that unbound PF largely determines the uptake of PF into myocardial tissue. It was shown that PF uptake into cardiac tissue was closely correlated to the concentration of AAG, and thus, the amount of free PF in the perfusate [Kates and Gilles, 1986]. As the concentration of AAG increased, the uptake of PF and subsequent
pharmacological activity were reduced [Kates and Gilles, 1986]. Thus, the influence of protein binding of PF may be important with respect to changes in AAG concentration and displacement of PF from AAG binding sites by other co-administered drugs. A similar situation may also exist for the active metabolite, 5-OH-PF, although this hypothesis remains untested.

Lipoprotein concentrations tend to vary significantly between subjects and even within a subject depending on disease and diet [Getz and Hay, 1979]. In addition, many genetic disorders exist where one or more sub-type of lipoproteins may be significantly elevated, as in familial hyperlipidemias [Brunzell and Miller, 1981]. Due to the variable nature of lipoprotein concentration, the influence of factors affecting PF and 5-OH-PF binding to lipoprotein complexes requires further study.

In addition to the basic science contribution of the presented work, this study may provide an estimate of the relative proportion of the free drug, (PF and 5-OH-PF) available for distribution into tissues (possible sites of pharmacological action) after the administration of PF. The protein profiles in human serum can change as a result of disease, stress, and environmental factors [Kremer et al., 1988]. Thus, the extent to which PF and 5-OH-PF bind to various serum proteins will provide useful information which may be used to predict binding in conditions where concentrations of binding proteins in serum may be altered.
1.6 AIMS

The purpose of the presented work is to determine the differences in the characteristics of the drug-protein interaction of PF and 5-OH-PF in human serum and to isolated human serum proteins by testing the following hypothesis:

THE IN VITRO PROTEIN BINDING CHARACTERISTICS OF PROPafenONE ARE DIFFERENT THAN THE BINDING CHARACTERISTICS OF IT'S ACTIVE METABOLITE, 5-HYDROXYP PropafenONE IN SERUM AND IN SOLUTIONS OF ISOLATED HUMAN SERUM PROTEINS.

In order to test the above hypothesis, the following list of objectives have been completed:

1. Establish an in vitro method for the accurate and reproducible determination of drug-protein binding of PF and 5-OH-PF in serum and in solutions of isolated serum proteins, and to identify possible sources of error.

2. Provide an accurate estimate of the binding of the metabolite, 5-OH-PF in serum with and without the presence of the parent compound, propafenone.

3. Determine the importance of AAG, HSA, and lipoproteins in the binding of PF in the presence of the metabolite 5-OH-PF, and the binding of 5-OH-PF in the presence of PF.
4. Examine the effect of probe displacers such as, disopyramide and ibuprofen, to better understand the binding interaction of PF and 5-OH-PF with serum, AAG and HSA.

5. Contrast the binding characteristics of PF and 5-OH-PF to AAG, HSA, HDL, LDL, and VLDL in buffer, and of PF and 5-OH-PF in serum.

6. Mathematically characterize the binding of PF and 5-OH-PF to individual binding proteins, and to try to model the binding of PF and 5-OH-PF in serum based on the models identified for the individual drug-protein interactions.

7. To characterize the uptake of PF and 5-OH-PF by red blood cells.
2. EXPERIMENTAL

2.1 MATERIALS AND SUPPLIES

2.1.1 DRUGS, METABOLITES, AND INTERNAL STANDARDS

PF hydrochloride, 5-OH-PF hydrochloride, Li-1115 hydrochloride, (internal standard, I.S. for PF quantitation) and Li-1548 hydrochloride (I.S. for 5-OH-PF quantitation) were supplied by Knoll Pharmaceuticals Canada Inc., Markham, Ont., Canada. Ibuprofen and disopyramide were obtained from Sigma Chemical Co., St. Louis, MO., U.S.A.

2.1.2 CHEMICALS AND REAGENTS

Heptafluorobutyric anhydride (HFBA) was purchased from Pierce Chemical Co. (Rockford, IL., U.S.A.). American chemical society reagent grade sodium hydroxide, monopotassium phosphate, disodium phosphate, sodium carbonate, potassium carbonate, glacial acetic acid, copper sulfate, and hydrochloric acid were obtained from BDH Chemicals, Toronto, Ont., Canada. Reagent grade (ACS) trichloroacetic acid was purchased from J.T. Baker Chemical Co., Phillipsburg, NJ., U.S.A. Barbital buffer pH 8.6, bromophenol blue, beta-mercaptoethanol, Sudan red No. 7B, sodium potassium tartrate, and phenol reagent were acquired from Sigma Chemical Co., St. Louis, MO., U.S.A. Sodium doedecyl sulfate, Coomasie blue, acrylamide, and N,N' methylene bis acrylamide were obtained from Bio Rad Laboratories, Mississauga, Ont. Glycerol, reagent grade was obtained from Anachema
Chemical Co., Richmond, B.C., Canada, and Sudan black was obtained from Aldridge Chemical Co., Milwaukee, WI., U.S.A.

2.1.3 PROTEINS

Crystallized and lyophilized essentially globulin free (<1 %) human serum albumin (A8763), essentially (< 0.005%) fatty acid free, globulin free (<1 %) human serum albumin (A3783), α-1-acid glycoprotein (G9885), and lipoprotein deficient human serum (S5519) were obtained from Sigma Chemical Co., St. Louis, MO., U.S.A. High density lipoproteins, low density lipoproteins, and very low density lipoproteins without ethylenediamine tetraacetic acid (EDTA) from human source were obtained as a special order from Calbiochem Co., San Diego, CA., U.S.A. Serum ultrafiltrate was prepared with the aid of an Amicon ultrafiltration device with an Amicon PM 30 membrane. Normal serum was added to the ultrafiltration device, and filtered with the aid of pressurized nitrogen as the driving force for filtration.

2.1.4 SOLVENTS

Pesticide grade toluene, methanol, hexane, dichloromethane, and isopropyl alcohol (distilled in glass) were purchased from Caledon Laboratories Ltd., Georgetown, Ont., Canada. Deionized distilled water was produced in the laboratory using a Milli-RO® Water System (Millipore Corp., Bedford, MA., U.S.A.).
2.1.5 GASES

Ultra high purity (UHP) hydrogen and argon/methane (95:5) were purchased from Matheson Gas Products Canada Ltd., Edmonton, Alta., nitrogen, and medical air U.S.P., from Union Carbide Canada Ltd., Toronto, Ont.

2.1.6 EQUILIBRIUM DIALYSIS CELLS

Plexi-Glass\textsuperscript{R} dialysis cells (1.0 mL) were used for equilibrium dialysis. Cellophane dialysis membrane "sacks" (Molecular Weight (M.W.) cutoff = 12,000 Daltons) were purchased from Sigma Chemical Co., St. Louis, MO., U.S.A.

2.1.7 ULTRAFILTRATION DEVICES

Ultrafiltration devices (MPS-1\textsuperscript{TM} Micropartition System) were purchased from Amicon Canada Ltd., Oakville, Ont. Each ultrafiltration device consisted of a single YMT membrane (molecular weight (M.W.) cutoff = 30,000 Daltons) and an O-ring, sealed between the sample reservoir and support base. The reservoir was provided with a cap to minimize sample evaporation and pH change due to loss of carbon dioxide. A removable filtrate collection cup was attached to the support base.
2.1.8 ELECTROPHORESIS

2.1.8.1 POLYACRYLAMIDE ELECTROPHORESIS GELS

Polyacrylamide gels were prepared by heating a mixture of acrylamide and N,N'-methylene bis-acrylamide; a catalyst was then added to the mixture. Stacking gels which are used to focus the protein sample before separation (3.5% acrylamide content), and the resolving gels which are used to separate the proteins on the basis of molecular weight (gradient of 5 to 20% acrylamide) were poured into the gel mold and allowed to cool.

2.1.8.2 AGAROSE ELECTROPHORESIS GELS

Universal EP Film agarose (470100) UNIVR was purchased from Ciba Corning Diagnostics Corp., Palo Alto, CA., U.S.A.

2.1.9 RADIAL IMMUNO DIFFUSION PLATES

NOR-PartigenR AAG radial immunodiffusion (RID) plates (12-well, volume = 5 µL per well) containing monospecific antiserum to human AAG in a ready-for use agarose-gel layer were purchased from Terochem Laboratories Ltd., Edmonton, Alta., Canada.

2.1.10 OTHER SUPPLIES

PyrexR disposable glass culture tubes (15 mL) were
purchased from Corning Glass Works, Corning, NY., U.S.A., and polytetrafluoroethylene (PTFE) lined screw caps from Canlab, Vancouver, B.C., Canada.

Venisystems™ Butterfly®-19 INT cannulae were purchased from Abbott Laboratories, Ltd., Montreal, Que., Canada. Vacutainer® blood collection tubes (with heparin and without additive) were obtained from Becton-Dickinson Canada Inc., Mississauga, Ont.

2.2 EQUIPMENT

2.2.1 GAS LIQUID CHROMATOGRAPH

A bonded-phase fused-silica capillary column, 25 m x 0.31 mm Internal diameter, was used for all gas liquid chromatography (GLC) analyses (stationary phase, cross-linked 5% phenylmethyl-silicone, film thickness 0.52 μm; phase ratio 150:1; Hewlett-Packard (HP), Palo Alto, CA, U.S.A.).

GLC analyses were performed on Model 5830A and 5840 HP (Palo Alto, CA., U.S.A.) gas-liquid chromatographs, equipped with Model 18835B capillary inlet systems, 63Ni electron-capture detectors, Model 18850A GC terminals for peak integration, and Model 7671A automatic samplers. A splitless injection mode was used, employing a fused-silica inlet liner with a small plug of silanized glass-wool 3 cm from the column end. Thermogreen® LB-2 septa (Supelco, Inc., Bellefonte, PA., U.S.A.), low-bleed at high inlet temperatures, were used. The septum was changed routinely to prevent leakage resulting
from repeated puncturing during automatic injection.

2.2.2 SPECTROPHOTOMETER

A Hewlett Packard 8452A diode array spectrophotometer equipped with a Hewlett Packard Vectra computer interface was used for all spectrophotometric measurements.

2.2.3 ELECTROPHORESIS EQUIPMENT

A Protein II electrophoresis tank with a model 1000/500 power supply was used to run the polyacrylamide gels (Bio Rad Laboratories, Mississauga, Ont., Canada). A Ciba Corning electrophoresis power supply and gel support were used to run the agarose gels.

2.2.4 OTHER

U.S.A.). An Advanced Wide Range Osmometer 3WII (Advanced Instruments Inc., Needham Heights, MI. U.S.A.) was used to measure the osmolality of the stock solutions.

2.3 PREPARATION OF STOCK AND REAGENT SOLUTIONS

2.3.1 DRUGS, METABOLITES, AND INTERNAL STANDARDS

PF hydrochloride was accurately weighed and dissolved in deionized distilled water. Serial dilutions were used to arrive at the final concentration of 100 ng/mL free base (11.07 mg of PF hydrochloride is equivalent to 10 mg of PF free base).

The metabolite, 5-OH-PF hydrochloride, was accurately weighed and dissolved in a mixture of methanol : deionized distilled water (1:9). Serial dilutions were used to arrive at a final concentration of 100 ng/mL free base (11.02 mg of 5-OH-PF hydrochloride is equivalent to 10 mg of 5-OH-PF free base).

Li-1115 hydrochloride was accurately weighed and dissolved in deionized distilled water. Serial dilutions were made to arrive at a final concentration of 200 ng/mL free base (11.11 mg of Li-1115 hydrochloride is equivalent to 10 mg of Li-1115 free base).

Li-1548 hydrochloride was accurately weighed and dissolved in deionized water. Using serial dilutions to give a final concentration of 200 mg/mL free base (11.06 mg of Li 1548...
hydrochloride is equivalent to 10 mg of Li-1548 free base).

All stock and diluted solutions were protected from sunlight by wrapping the glass containers in aluminum foil and were stored them at 4 °C after preparation, for up to four months.

2.3.2 REAGENTS AND SOLUTIONS

Triethylamine (0.003 M) was prepared by diluting triethylamine with toluene. Four or five pellets of NaOH were added to the solution.

Sodium hydroxide (NaOH) 1 M and 5 M solutions were prepared by dissolving NaOH pellets in deionized distilled water.

Sodium carbonate (Na₂CO₃) 0.1 M solution and potassium carbonate (K₂CO₃) 5 M solution were prepared by dissolving Na₂CO₃ and K₂CO₃ powder in deionized distilled water, respectively.

Hydrochloric acid (HCL) 1 M was prepared by diluting ACS reagent grade concentrated (37% W/W) HCL in deionized distilled water.

Phosphate buffer (pH 7.4) was prepared using the following procedures. Monopotassium phosphate (KH₂PO₄, 2.69 G), disodium phosphate (Na₂HPO₄, 11.40 G), and sodium chloride (NaCl 1.62 G) were accurately weighed and dissolved in deionized distilled water to a final volume of 1000 mL to yield a phosphate buffer of 0.10 M. The pH
of the final solution was checked and adjusted, if necessary, to 7.4 by using aliquots of 1.0 M NaOH or HCl.

Phosphate buffer (pH 6.0) was prepared by dissolving 2.28 g of KH$_2$PO$_4$ in deionized distilled water to a volume of 250 mL (solution 1) and 0.95 g Na$_2$HPO$_4$ to a volume of 100 mL (solution 2). The final buffer solution was prepared by combining solutions 1 and 2 (90 mL of solution 1 and 10 mL of solution 2).

Sodium carbonate (2 g) was dissolved in 100 mL of 0.1 M NaOH to yield a 2% sodium carbonate solution in 0.1 M NaOH. Copper sulfate (CuSO$_4$·5H$_2$O; 1 g) and sodium potassium tartrate (2 g) were dissolved in 100 mL distilled deionized water. Phenol reagent was diluted 1:1 with distilled deionized water at each use.

2.3.3 PROTEIN SOLUTIONS

Human serum albumin (HSA) and essentially (< 0.005%) free fatty acid free albumin (4.5 g) were dissolved in 100 mL of pH 7.4 isotonic phosphate buffer to yield a final concentration of 45 mg/mL which would approximate physiological concentrations of HSA. α-1-acid glycoprotein (AAG) (0.09 g) was dissolved in 100 mL of pH 7.4 isotonic phosphate buffer to yield a final concentration of 0.9 mg/mL which would approximate physiological concentrations of α-1-acid glycoprotein.

High density lipoproteins (HDL), low density lipoproteins
(LDL), and very low density lipoproteins (VLDL) in isotonic pH 7.2 Krebs buffer were diluted from the original concentration with 0.1 M phosphate pH 7.4 buffer to yield final physiological concentrations of 3.0 mg/mL, 3.0 mg/mL, and 1.5 mg/mL of HDL, LDL, and VLDL, respectively.

Bovine serum albumin (BSA) 100 mg was dissolved in 100 mL of distilled deionized water to provide a final concentration of 1 mg/mL.

2.3.4 ESTABLISHED TECHNIQUES USED IN RESEARCH

2.3.4.1 GAS LIQUID CHROMATOGRAPHY OF PROPafenONE AND 5-HYDROXYPROPafenONE

Sensitive and selective capillary gas liquid chromatographic techniques employing electron capture detection [Chan et al., 1988; Chan et al., 1989a] were used to quantify PF and 5-OH-PF. The PF assay employed a liquid-liquid extraction using toluene as the extraction solvent. Following extraction, PF samples were evaporated under a gentle stream of nitrogen gas at 40 °C. The samples were then reconstituted with triethylamine in toluene, and derivatized with heptafluorobutyric anhydride. These derivatized samples were then injected into the gas chromatograph and quantified. The 5-OH-PF assay employed similar techniques, but differed in the extraction solvent [toluene 7 : methylene chloride 3 : isopropyl alcohol 1 vs. toluene] and the bases [0.1 M sodium carbonate vs. 1.0
M NaOH, and 5 M potassium carbonate vs. 5 M NaOH] used.

2.3.4.2 LOWRY PROTEIN ASSAY

The Lowry protein assay method was used to quantify proteins present in samples used in the drug protein binding experiments. A standard curve using bovine serum albumin (BSA) was prepared (0 - 100 μg/0.1 mL BSA). The appropriate dilutions of the protein samples were made and 0.1 mL of these solutions were pipetted into a clean culture tube. A 1.0 mL aliquot of 0.01% CuSO₄·5H₂O, 0.02% sodium potassium tartrate, 2.0% NaCO₃ in 0.1 M NaOH was added, then the solution was vortexed. Following a 15 minute incubation step at room temperature, 0.1 mL of a 1.0 M phenol solution was added and the solution immediately vortexed. This mixture was allowed to stand at room temperature for 30 minutes. The samples were then pipetted into optical glass cuvettes and read at 650 nm on the spectrophotometer.

2.3.4.3 POLYACRYLAMIDE GEL ELECTROPHORESIS

Prepared gels were placed into the electrophoresis tank, and submerged in electrophoresis buffer (0.1 M sodium phosphate buffer pH 7.0 with 1% sodium dodecyl sulfate). Appropriate amounts of protein (25.0 μg) were pipetted into sample buffer (20 parts [0.5 M phosphate buffer pH 7.0, 1.0% sodium dodecyl sulfate, and 40% Glycerol]: 5 parts [bromophenol blue solution]: and 5 parts [beta-mercaptoethanol solution]), the samples were then heated for 90
seconds, cooled, and carefully applied to the stacking gel. The gels were run at a constant current of 25 mA until the tracking dye was approximately 2 cm from the bottom of the gel. The samples were then removed from the gel apparatus, and placed in staining solution (0.25% Coomassie Brilliant Blue R250, 45% methanol, 9% glacial acetic acid, and 45% distilled water). After 1.0 hour in the staining solution, the gel was placed into the first destaining solution (45% methanol, 10% glacial acetic acid, and 45% distilled water) and gently mixed. After 1.0 hour the gels were placed into the second destaining solution (20% methanol, 5% acetic acid, and 75% distilled water). The gel remained in this solution for 24 hours until the background of the gel was relatively clear.

2.3.4.4 LIPOPROTEIN AGAROSE ELECTROPHORESIS

Aliquots of 5.0 μL of sample lipoprotein solutions were applied into the prepared wells on the agarose gel along with one control lane containing only bromophenol blue. The plate was then put into the electrophoresis apparatus, and the buffer wells filled with 0.05 M barbital pH 8.5 buffer. Electrophoresis was carried out for 20 minutes at a constant voltage of 90 volts. The agarose plate was then removed and washed for 5 minutes with 1% glycerol in 55% ethanol. After the glycerol wash, the proteins were fixed at 70-80 °C for 1.5 to 2 hours. Following this, the plates were cooled and stained for approximately 10 minutes with a stainer (200 mL stock solution [225 mg Flat B Red in 946 mL ethanol], 40 mL 0.1 M NaOH, and 8 drops of Triton X-100). Following staining, plates were rinsed in 2% glycerol for 30
seconds, and dried at 70-80 °C for 15 minutes.

2.4 PROTEIN BINDING OF 5-HYDROXYPROPafenONE AND PROPafenONE

2.4.1 PRELIMINARY EXPERIMENTS

2.4.1.0 PREPARATION OF EQUILIBRIUM DIALYSIS DEVICES

Cellophane dialysis membranes were immersed in boiling distilled water for 1 hour, and then soaked in isotonic phosphate buffer (pH 7.4) overnight at 4 °C. The membranes were then cut and mounted on the plastic equilibrium dialysis apparatus. Extreme care was taken to avoid contact of the membrane with possible contaminating surfaces (fingers, lab bench, dirty tools, etc.). After placement of the membrane using forceps, the two halves of the matched cells were then tightly fastened together to avoid leakage during the dialysis experiments. Equal volumes of the desired solutions (serum, buffer, or protein solutions) were then added to both sides of the membrane, and the cells sealed. The cells were then immersed and rotated at 14 rpm in the water bath at 37 °C for eight hours. Following dialysis, both buffer and serum were transferred to clean test tubes, and frozen.

2.4.1.1 TIME TO EQUILIBRIUM

Isotonic phosphate buffer (pH 7.4) containing 0.5 μg/mL 5-OH-PF was dialyzed against an equal volume (0.8 mL) of blank serum at
37 °C for 2, 4, 6, 8, and 10 hours. The concentration of 5-OH-PF in the buffer and serum side of the cell was measured, using a sensitive and selective GLC-ECD method. The free fraction was then plotted against time. Equilibration was established when the plot of free fraction reached a plateau (i.e., the free fraction fails to decrease further). The time to attain equilibrium was evaluated at low (0.1 μg/mL) and high (45.0 μg/mL) concentrations of 5-OH-PF. Measurements of pH were made in both the serum and the buffer following dialysis.

2.4.1.2.1 NON-SPECIFIC BINDING OF 5-HYDROXYPROPafenONE TO EQUILIBRIUM DIALYSIS CELLS AND MEMBRANES.

The extent of adsorption of 5-OH-PF to the surface of the equilibrium dialysis apparatus was determined by measuring the concentration of 5-OH-PF spiked buffer before and after dialysis. Following dialysis at 37 °C for 8 hours, aliquots of buffer were removed, analyzed by the above described GLC-ECD method, and compared to undialyzed spiked buffer. Non-specific binding experiments were conducted at 0.1, 0.2, 0.5, 1.0, 5.0, and 25.0 μg/mL. The percentage of non-specific adsorption was then calculated by equation 10:

\[
\text{% NON-SPECIFIC BINDING} = \frac{(\text{CONC. BEFORE} - \text{CONC. AFTER})}{\text{CONC. BEFORE}}
\]

where CONC. BEFORE and CONC. AFTER are the concentrations of 5-OH-PF of spiked buffer before and after dialysis, respectively.
2.4.1.2.2 NON-SPECIFIC BINDING OF PROPAFENONE AND 5-HYDROXYPROPafenone TO AMICON MPS-1 ULTRAFLTRATION DEVICES.

Isotonic phosphate pH 7.4 buffer spiked with PF and 5-OH-PF was added to the ultrafiltration devices. The devices were then capped and placed into an ultracentrifuge, equilibrated to a temperature of 25 °C, and spun for 20 minutes at 2000 g at a fixed angle of 18°. Aliquots from the ultrafiltration cup were analyzed for both PF and 5-OH-PF. The percentage loss was calculated using equation 1, where CONC. BEFORE and CONC. AFTER are the concentrations of spiked buffer before and after ultrafiltration.

2.4.3 pH DEPENDENT BINDING OF PF AND 5-HYDROXYPROPafenONE

2.4.3.1 pH DEPENDENT BINDING OF PROPAFENONE IN SERUM

Isotonic phosphate buffers ranging from a pH of 6.0, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, to 9.0 were prepared and spiked with 5-OH-PF or PF to arrive at a final concentration of 0.2 μg/mL. The pH of the serum was adjusted by the drop-wise administration of 0.1 N sodium hydroxide, or 0.1 N hydrochloric acid. The pH of both the buffer and serum solutions were measured before and after dialysis at 37 °C for 8 hours. Following dialysis, aliquots of both the buffer and serum compartments of the dialysis cell were removed and assayed for 5-OH-PF or PF by GLC-ECD. The free fraction of PF and 5-OH-PF in serum was plotted against the serum pH after dialysis. Total serum protein was
also measured using a Lowry protein assay method described above.

2.4.3.2 pH DEPENDENT BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE IN ISOLATED PROTEIN SOLUTIONS (ALBUMIN AND AAG)

The pH dependent binding of PF and 5-OH-PF to HSA and AAG was established. Both AAG and HSA were dissolved in isotonic phosphate buffer of varying pH (7.0, 7.2, 7.4, 7.6, and 7.8) at the physiological concentrations described in section 2.3.3. These solutions were then spiked with PF or 5-OH-PF to result in concentrations of 2.0 μg/mL and 0.5 μg/mL, respectively. The protein solutions were then dialyzed against blank isotonic phosphate buffer of a corresponding pH. After dialysis was completed, samples were removed, pH measured, and the post-dialysis buffer and protein compartments assayed for both PF and 5-OH-PF. The free fraction was calculated and plotted as a function of pH. Total protein and concentration of AAG were measured by the Lowry method and radial immunodiffusion plates, respectively.

2.4.3.3 pH PARTITION COEFFICIENT IN TOLUENE AND HEXANE OF PROPafenONE AND 5-HYDROXYPROPafenONE.

Buffers were prepared at various pH (7.0, 7.2, 7.4, 7.6, 7.8, and 8.0) spiked with either PF or 5-OH-PF. Aliquots of spiked buffer (1.5 mL) were added to 5 mL of either hexane or toluene in a clean test tube. These tubes were then capped and mixed for 20 minutes. Following mixing, organic and aqueous layers were separated,
and transferred into clean test tubes. PF and 5-OH-PF were measured in both the organic, and aqueous layers. The disappearance of drug from the aqueous layer was plotted vs. pH for the respective solvent and drug.

2.4.4 BUFFER STRENGTH

Isotonic phosphate buffers at various ionic strengths were prepared (0.067, 0.100, 0.150 M). Each buffer was spiked to allow a final concentration of 0.5 μg/mL 5-OH-PF. Equal volume (0.8 mL) aliquots of buffer and serum were applied to opposite sides of the dialysis apparatus, and then dialyzed as described above. After 8 hours of dialysis, aliquots of both the serum and buffer compartments were assayed for 5-OH-PF. The free fractions (free/total drug concentration) were compared at various buffer strengths using a non-parametric test (Mann Whitney U-Test) with a significance level of p=0.05.

2.4.5 CHEMICAL DEGRADATION OF 5-HYDROXYPROPafenone DURING DIALYSIS

Aliquots of serum and buffer were spiked with 5-OH-PF to yield a final concentration of 0.5 μg/mL. A sample was removed before incubation to serve as a control sample. The rest of the samples were incubated for 1, 2, 4, 7.4, and 8 hours at 37 °C. Aliquots from the various samples were removed and assayed for 5-OH-PF. The concentrations of 5-OH-PF were then compared to concentration of 5-OH-PF from the sample removed before the incubation, using the Kruskall-
Wallis test with a significance level of $p=0.05$.

2.4.6 VOLUME SHIFTS

An aliquot (0.8 mL) of buffer and an equal volume of serum were added to the dialysis cell. This was then dialyzed for 8 hours at 37°C. Following the dialysis, the total volume on each side of the cell was removed, using a 1.0 mL Hamilton syringe. The volumes on each side of the cell were carefully measured, and compared to the volume prior to dialysis (a correction for the dead volume in the needle was made before comparison). These results were then compared to a control experiment in which only buffer was added to both sides of the cell.

2.4.7 OSMOLARITY OF 5-HYDROXYPROPafenONE SOLUTIONS

The osmolarity of phosphate pH 7.4 buffer spiked with various concentrations of 5-OH-PF (0.1 to 45 μg/mL) was determined.

2.4.8 LOSS OF PROTEIN DURING DIALYSIS

Measurements of total protein and AAG in serum, measurements of total protein in an albumin solution, and measurements of AAG in a solution of AAG were made before and after 8 hours of dialysis at 37 °C to assess the loss of proteins during equilibrium dialysis.
2.4.9 TEST FOR DIALYSIS MEMBRANE INTEGRITY FOLLOWING DIALYSIS

To determine the integrity of the dialysis cell membranes following dialysis, small aliquots of post-dialysis buffer were added to a solution of 3% trichloroacetic acid. If precipitation was observed the samples were discarded due to the presence of protein in the buffer.

2.5 BINDING OF 5-HYDROXYPROPafenONE IN HUMAN SERUM

2.5.1 BINDING IN NORMAL SERUM

Blood from seven healthy male Europids was collected into red top vacutainers (no additives) from the median cubital vein with the aid of an indwelling Butterfly-19R Int cannula. Extreme care was taken to avoid contact of the blood with the red plastic stopper. The blood was allowed to clot by standing at room temperature for 30 minutes. The blood was then centrifuged for 15 minutes at 2000 rpm at a fixed angle of 20°. The serum was then carefully removed with borosilicate pipettes, and transferred to clean test tubes. The total protein concentration and the concentration of AAG was determined from representative samples from each volunteer. The tubes were capped with polytetrafluoroethylene lids and frozen for a maximum of one week before use. Aliquots of serum were then thawed and added to the serum side of the equilibrium dialysis apparatus. Spiked buffer was added to the other side of the dialysis cell at concentrations of 0.1, 0.2, 0.5, 1.0, 5.0, 10.0, 25.0, and 45 μg/mL. The cells were then sealed,
placed in a water bath at 37 °C, and rotated for 8 hours at 14 rpm. After the dialysis had been completed, aliquots of both buffer and serum were removed and analyzed for 5-OH-PF with GLC-ECD. The free fraction of 5-OH-PF at total drug concentrations (the concentration in the serum side of the dialysis cell) was reported.

2.5.2 ROSENTHAL PLOTS OF 5-HYDROXYPROPAFENONE BINDING IN SERUM

Data obtained from the above set of experiments was plotted by the method of Rosenthal (e.g. binding ratio [bound/free] vs. concentration of bound drug). The computer program ENZFITTER (Elsevier-BIOSOFT) was used to determine the best non-linear regression fit from the untransformed data (Bound drug vs. Free drug). From this information, binding parameters for 5-OH-PF such as number of binding sites, association constants, and the binding capacity of serum proteins could be determined.

2.5.3 PROTEIN BINDING OF 5-HYDROXYPROPAFENONE IN SERUM DETERMINED BY EQUILIBRIUM DIALYSIS IN THE PRESENCE OF PROPAFENONE

The effect of the addition of PF on 5-OH-PF serum binding was assessed by measuring the binding of 5-OH-PF in serum from healthy male volunteers spiked with PF (2.0 μg/mL) and 5-OH-PF (0.5 μg/mL). Aliquots of spiked serum were added to the dialysis cells and dialyzed for 8 hours at 37 °C against blank buffer. Following dialysis, aliquots of both serum and buffer were analyzed for PF and 5-OH-PF, using the GLC-ECD method described previously. The free fraction of
5-OH-PF in the presence of PF was calculated and compared to the control free fraction of 5-OH-PF alone, using the non-parametric Mann-Whitney U-test, at a significance level of p=0.05.

2.5.4 BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE USING THE METHOD OF ULTRAFILTRATION

Serum (0.8 mL) samples from healthy male volunteers were spiked with PF and 5-OH-PF to yield concentrations of 2.0 μg/mL and 0.5 μg/mL, respectively. The samples were then placed into the ultrafiltration devices. The ultrafiltration devices were then placed into the ultracentrifuge, equilibrated to a temperature of 25 °C, and rotated at 2000 g for 20 minutes at a fixed angle of 18°. The resulting filtrate volume was approximately 0.5 mL. Aliquots of spiked serum before centrifugation, and ultrafiltrate were then assayed for both PF and 5-OH-PF. These results were compared to results obtained by equilibrium dialysis.

2.6 BINDING OF 5-HYDROXYPROPafenONE AND PROPafenONE TO ISOLATED PROTEINS

2.6.1 BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE TO HSA, AND AAG

The binding of PF and 5-OH-PF to pure HSA, pure human AAG, and both HSA plus AAG was assessed with protein dissolved in isotonic phosphate pH 7.4 buffer, and serum ultrafiltrate. HSA, AAG, and AAG
plus HSA were dissolved in both buffer and human serum ultrafiltrate at physiological concentrations. The protein solutions were then spiked with PF and 5-OH-PF to yield a final concentration of 2.0 μg/mL and 0.5 μg/mL, respectively. Aliquots (0.8 mL) of the above solutions were then dialyzed against blank isotonic phosphate pH 7.4 buffer for 8 hours at 37 °C. Following dialysis, the buffer and serum samples were removed, pH measured, and the concentrations of PF and 5-OH-PF measured as discussed above. The free fraction of PF and 5-OH-PF in each treatment case was calculated. Differences in binding between human serum ultrafiltrate and buffer were compared using the two sample T-test with a significance level of p=0.05. Concentrations of total protein and AAG were measured.

2.6.2 BINDING TO PROPAFENONE AND 5-HYDROXYPROPafenONE TO ALBUMIN AND FREE FATTY ACID FREE ALBUMIN.

Binding of PF and 5-OH-PF to both albumin and (< 0.005%) free fatty acid free albumin were assessed. Both albumin and free fatty acid free albumin were dissolved in isotonic phosphate pH 7.4 buffer to yield physiological concentrations. These solutions were then spiked with both PF and 5-OH-PF to yield final concentrations of 2.0 μg/mL and 0.5 μg/mL, respectively. Aliquots of each solution were dialyzed against an equal volume of blank isotonic phosphate pH 7.4 buffer for 8 hours at 37 °C. Following dialysis, aliquots of buffer and serum were removed from the dialysis cells, pH, and the concentration of PF and 5-OH-PF were measured. Statistical comparisons were made using a two sample T-test with a significance
level of \( p=0.05 \). Concentrations of total protein were measured by the method of Lowry.

2.6.3 BINDING OF PROPAFENONE AND 5-HYDROXYPROPAFENONE TO LIPOPROTEIN DEFICIENT SERUM

The binding of PF and 5-OH-PF to control serum and lipoprotein deficient serum was measured. Both control serum and lipoprotein deficient serum were spiked with PF and 5-OH-PF to yield final concentrations of 2.0 \( \mu g/mL \) and 0.5 \( \mu g/mL \), respectively. Equal volumes of spiked serum and lipoprotein deficient serum were dialyzed against blank isotonic phosphate pH 7.4 buffer for 8 hours at 37 °C. Following dialysis, samples were removed, pH measured, and the concentrations of PF and 5-OH-PF were measured in the samples, as described previously. Statistical comparisons were made using the two sample T-test with a significance level of \( p=0.05 \). Concentrations of total protein and AAG were measured, as described previously.

2.6.4 ELECTROPHORESIS OF ISOLATED PROTEIN SOLUTIONS

Purity of the isolated protein solutions was assessed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). Samples of AAG, HSA, HSA + AAG, lipoprotein deficient serum, and normal serum were subjected to SDS PAGE to both confirm and assure protein purity.
2.6.5 DRUG-DRUG DISPLACEMENT OF PROPafenONE AND 5-HYDROXYPROPafenONE FROM SERUM PROTEINS BY DISOPYRAMIDE, AND IBUPROFEN

The effect on the binding of PF and 5-OH-PF by the addition of displacers such as the acidic drug, ibuprofen, and the amine drug, disopyramide, was assessed. Serum spiked with both PF (2.0 µg/mL) and 5-OH-PF (0.5 µg/mL) with the addition of therapeutic concentrations of either ibuprofen (50 µg/mL) or disopyramide (8.0 µg/mL) was dialyzed against blank isotonic pH 7.4 phosphate buffer for 8 hours. Aliquots of serum and buffer were removed and assayed for PF and 5-OH-PF by GLC-ECD. The free fraction of PF and 5-OH-PF were calculated and compared to control samples using the Kruskall-Wallis test with a significance level of p=0.05.

2.6.6 DRUG-DRUG DISPLACEMENT OF PROPafenONE AND 5-HYDROXYPROPafenONE FROM HSA AND AAG BY DISOPYRAMIDE AND IBUPROFEN

Displacement of PF and 5-OH-PF from individual proteins was determined by using two probe displacing agents disopyramide, and ibuprofen. Protein samples, HSA and AAG, were dissolved in isotonic phosphate pH 7.4 buffer at physiological concentrations. Both PF and 5-OH-PF were then added to give final concentrations of 2.0 µg/mL and 0.5 µg/mL. The samples were spiked with either disopyramide 8.0 µg/mL, or ibuprofen 50 µg/mL. Aliquots of protein solutions containing a mixture of PF and 5-OH-PF mixture, and either ibuprofen or disopyramide were then added to the dialysis cells and dialyzed against blank isotonic phosphate pH 7.4 buffer for 8 hours at 37 °C.
Following dialysis, samples were removed from the dialysis cells, pH measured, and the concentration of PF and 5-OH-PF measured. Concentrations of total protein and AAG were assessed.

2.7 CHARACTERIZATION OF BINDING OF PROPAFENONE AND 5-HYDROXYPROPafenone IN PROTEIN SOLUTIONS

2.7.1 CHARACTERIZATION OF BINDING TO POOLED HUMAN SERUM, AAG, HSA, HDL, LDL, AND VLDL SOLUTIONS

Protein binding of PF and 5-OH-PF was characterized by equilibrium dialysis in pooled human serum, and in physiological concentrations of AAG, HSA, and human lipoproteins [HDL, LDL, and VLDL] dissolved in isotonic phosphate pH 7.4 buffer. Buffer was spiked with varying concentrations of either PF [0.25, 0.50, 1.0, 2.0, 5.0, 10.0, 25.0, 50.0, 75.0, and 100.0 μg/mL] or 5-OH-PF [0.20, 0.50, 0.75, 1.00, 2.00, 5.00, 10.0, 20.0, 30.0, and 45.0 μg/mL]. Spiked buffer was dialyzed against equal volumes (0.8 mL) of blank protein solutions at physiological concentrations previously discussed. Following 8 hours of equilibrium dialysis at 37 °C, samples were removed, pH measured, and concentrations of either PF or 5-OH-PF measured. Binding ratio (bound/free) was plotted against concentration of bound drug to obtain a Rosenthal plot for the binding of PF or 5-OH-PF to each protein solution described above. The free fractions of PF and 5-OH-PF were plotted against the respective total drug or metabolite concentration measured. Analysis of binding parameters of PF or 5-OH-PF to each protein solution was carried out
by non-linear regression analysis of several defined binding models (see section 2.8.2 BINDING PARAMETER MODELS AND DATA FITTING) using the computer program ENZFITTER\textsuperscript{R}. Concentrations of total protein and AAG were assessed as described previously.

2.7.2 ELECTROPHORESIS OF LIPOPROTEINS

To ensure that the composition of the lipoprotein complex would not deteriorate during dialysis, aliquots of lipoprotein were subjected to electrophoretic separation on an agarose gel before and after the dialysis procedure. Lipoproteins (LDL, VLDL, and HDL) were applied to the agarose gel and run as described previously.

2.8. DATA ANALYSIS

2.8.1 STATISTICAL ANALYSIS OF DATA.

The data in these experiments were analyzed by a number of different statistical techniques. These statistical techniques are stated where applied. Where assumptions of normal distribution, and equal variance could not be demonstrated non-parametric tests were used. The non-parametric equivalent for the two sample t-test is the Mann-Whitney U-test, and for the one-way analysis of variance (ANOVA), is the Kruskall-Wallis test. If non-parametric tests are inadvertently used, and the assumptions of normality, and equal variance are met, they are approximately 95% as powerful as their parametric counterparts [Zar, 1987].
2.8.2 BINDING PARAMETER MODELS AND DATA FITTING

Several models, derived from first principles of protein-ligand interactions (equation 1), were used to model the binding observed for 5-OH-PF in serum samples of healthy volunteers, and for both PF and 5-OH-PF in solutions of isolated proteins.

\[
[DP] \rightleftharpoons [D] + [P] \quad (1)
\]

The simplest binding model used was that of total non-specific binding (Equation 11), followed by 1 site-specific binding (Equation 12), 1-site specific + non-specific binding (Equation 13), and finally 2 site-specific ligand binding (Equation 14).

\[
[Db] = Kns*[Df] \quad (11)
\]

The equation for non-specific binding (equation 11) is merely a simplification of the equation for the 1 site binding model. That is, if the affinity for binding of a drug to this site is very low, equation 12 collapses to the product of the binding affinity and the binding capacity (i.e. Kns = Ka*NP1) [Glasson et al., 1980].

\[
[Db] = \frac{NP1*Ka1*[Df]}{1 + Ka1*[Df]} = NP1*Ka1*[Df] \text{ if } Ka1 << 1 \quad (12)
\]

\[
[Db] = \frac{(NP1*Ka1*[Df]) + Kns*[Df]}{1 + Ka1*[Df]} \quad (13)
\]

\[
[Db] = \frac{(NP1*Ka1*[Df]) + (NP2*Ka2*[Df])}{(1 + Ka1*[Df]) (1 + Ka2*[Df])} \quad (14)
\]
Where \([Db]\) is the molar bound drug concentration, \([Df]\) is the molar free drug concentration, \(NP1\) is the capacity of the first drug binding site (molar), \(Ka1\) is the association constant of the first binding site (molar\(^{-1}\)), \(NP2\) is the capacity of the second binding site (molar), and \(Ka2\) is the association constant of the second binding site (molar\(^{-1}\)).

The non-transformed binding data (bound vs. free) was fit with the non-linear curve fitting computer program ENZFITTER\(^R\) to one of the above binding equations. The "goodness of fit" was determined from the standard deviation of the estimates, and the random equal distribution of the y-axis residuals.

Non-linear curve fitting with the ENZFITTER\(^R\) program employs the enhanced algorithm of Marquart [Leatherbarrow, 1987] to fit the data. The ENZFITTER\(^R\) program also has robust weighting. Robust weighting allows extreme outliers to be eliminated based on statistical principles rather than subjective elimination. Robust weighting employs the algorithm of Mosteller and Tukey [Leatherbarrow, 1987] to statistically reduce the importance of outliers in the data undergoing non-linear regression. For qualitative purposes, data was plotted by the method of Rosenthal, as opposed to Scatchard. This is due to the variety of different proteins present in serum making the use of total protein concentration, as used in Scatchard, incorrect.

2.8.3 MATHEMATICAL BINDING RECONSTITUTION STUDIES
Binding of PF and 5-OH-PF in serum will be estimated based on the sum of the binding contributed by each protein as in equation 15:

\[
[\text{Db}]_{\text{serum}} = \sum_{i=1}^{n} [\text{Db}]_{n}
\]  

(15)

where \([\text{Db}]_{\text{serum}}\) is the theoretically calculated binding of either PF or 5-OH-PF from the sum of the contributions of binding by individual binding proteins. The term \(n\) is the number of proteins contributing to binding of drug, and \([\text{Db}]_{n}\) is the binding contribution due to each individual protein. The next step is to systematically subtract the contribution of individual proteins, and then calculate the concentration of bound drug (see equation 16).

\[
[\text{Db}]_{\text{serum}} = \sum_{i=5}^{n-1} [\text{Db}]_{n}
\]  

(16)

where \([\text{Db}]_{\text{serum}}\) is the concentration of bound drug calculated for a specific concentration of free drug \([\text{Df}]\), as the binding contribution of individual constituent proteins is systematically subtracted (in the order VLDL, LDL, HDL, HSA). Finally \([\text{Db}]_{\text{serum}}\), \([\text{Db}]_{\text{serum}}\), \([\text{Db}]_{\text{serum}}\), \([\text{Db}]_{\text{serum}}\), and \([\text{Db}]_{\text{serum}}\) are plotted as a function of free drug in order to assess the relative importance of each protein to the overall binding of PF and 5-OH-PF.

2.9 RED BLOOD CELL DISTRIBUTION
2.9.1 PREPARATION OF RED BLOOD CELLS

Blood was collected from healthy volunteers into red top vacutainers. The caps were immediately removed, whereupon 2 mL aliquots of blood were transferred to clean test tubes, and diluted with 8 mL of isotonic pH 7.4 phosphate buffer. The contents of the tubes were then gently mixed for 10 minutes, and the tubes were centrifuged at -1000 g for 5 minutes. The top aqueous layer was removed and discarded, another 8 mL of fresh isotonic pH 7.4 phosphate buffer was added, and the procedure was repeated. This washing procedure was repeated for a total 3 washes. During the final wash step, the cells were spun for 20 minutes at -1000 rpm. The aqueous layer was then removed, leaving packed red cells. The red cells (0.55 mL) were reconstituted in isotonic pH 7.4 phosphate buffer (0.45 mL) and gently mixed for 5 minutes.

2.8.2 TIME TO DISTRIBUTION EQUILIBRIUM FOR PROPafenone AND 5-HYDROxyPROPafenone UPTAKE INTO HUMAN RED BLOOD CELLS

Reconstituted red blood cell suspensions were spiked with PF to give a final concentration of 2.0 μg/mL and 5-OH-PF to give 0.5 μg/mL. These samples were immediately placed in a water bath at 37 °C for various time periods (2, 4, 6, ...30 minutes) to assess the time required to attain distributional equilibrium. The samples were then immediately centrifuged for 2 minutes, and the supernatant removed. The red blood cells were then hemolyzed by adding distilled water, and vortexing the mixture. The supernatant and hemolyzed red cells were
analyzed for both PF and 5-OH-PF. The ratio of drug contained in red blood cells (concentration of drug in red cells/concentration of drug in supernatant) was calculated and plotted with respect to time.

2.8.3 UPTAKE OF PROPafenone AND 5-HYDROXYPROPafenONE BY HUMAN RED BLOOD CELLS.

Red blood cell suspensions were prepared in either isotonic phosphate pH 7.4 buffer, or serum. Whole blood was also used to estimate drug uptake by red blood cells. These suspensions were then spiked with PF and 5-OH-PF to yield final concentrations of 2.0 μg/mL and 0.5 μg/mL, respectively. These samples were then incubated at 37 °C for 30 minutes. The samples were then removed and centrifuged for 10 minutes at -1000 g. Aliquots of supernatant and packed red blood cells were then analyzed for PF and 5-OH-PF. The ratio of drug concentration in red blood cells over the drug concentration in the supernatant were calculated as in equation 8:

\[
\text{Red blood cell uptake ratio} = \frac{[\text{D red blood cell}]}{[\text{D supernatant}]} \quad (17)
\]

Where \([\text{D red blood cell}]\) is the concentration of either PF or 5-OH-PF present in red blood cells, and \([\text{D supernatant}]\) is the drug concentration of the supernatant. This ratio was calculated for PF and 5-OH-PF for each treatment.
3. RESULTS

3.1 PRELIMINARY IN VITRO PROTEIN BINDING EXPERIMENTS FOR PROPafenONE AND 5-HYDROXYPROPafenONE

3.1.1 TIME TO EQUILIBRIUM

The time to reach binding equilibrium was established to occur between 6 and 7 hours (Figure 2a and 2b) when both high (45.0 μg/mL) and low (0.1 μg/mL) concentrations of 5-OH-PF were dialyzed against 0.1 M isotonic phosphate buffer. Eight hours was chosen as the optimum dialysis time to attain equilibrium. The pH of the serum compartment following dialysis did not vary significantly over the time required to reach equilibrium.

3.1.2 NON-SPECIFIC BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE TO EQUILIBRIUM DIALYSIS CELLS AND MPS-1 ULTRA-FILTRATION DEVICES

The extent of non-specific binding of 5-OH-PF to both the dialysis cell and the membrane was determined over a range of concentrations. The extent of non-specific binding of 5-OH-PF was high over the entire concentration range studied (Table 1). The total loss to the cell with the membrane used for equilibrium dialysis was 28.2 ± 6.8% (Mean ± SD).
Figure 2. Time required to attain equilibrium for the equilibrium dialysis of 5-hydroxypropafenone.

a) 0.1 μg/mL of 5-hydroxypropafenone
b) 45.0 μg/mL of 5-hydroxypropafenone

The pH of the serum compartment of the dialysis cell following dialysis is presented as a bar graph. (pH ± SD)
TABLE 1. COMPARISON OF NON-SPECIFIC BINDING OF 5-HYDROXYPROPANEONE AND PROPANEONE TO ACRYLIC EQUILIBRIUM DIALYSIS APPARATUS FOLLOWING INCUBATION FOR 8 HOURS AT 37°C AND MPS-1 ULTRAFLTRATION DEVICES AT 25°C.

<table>
<thead>
<tr>
<th>CONCENTRATION (µg/mL)</th>
<th>5-HYDROXYPROPANEONE UTLRAFLTRATION</th>
<th>PERCENT LOST</th>
<th>EQUILIBRIUM</th>
<th>PERCENT LOST</th>
<th>MPS-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PERCENT LOST</td>
<td>EQUILIBRIUM</td>
<td>PERCENT LOST</td>
</tr>
<tr>
<td>0.10</td>
<td>25.6 ± 15.6%</td>
<td>-</td>
<td>25.0 ± 7.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>32.0 ± 9.6%</td>
<td>25.0 ± 7.2%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>36.5 ± 3.0%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td>28.5 ± 9.2%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.00</td>
<td>18.4 ± 2.4%</td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

CONCENTRATION (µg/mL) PROPANEONE

|                       |                                       |              | 19.8 ± 5.3% |              |       |
| 2.00                  |                                       |              |              |              |       |
| 10.00                 |                                       |              |              |              |       |

NOTE: EXPERIMENTS WERE CARRIED OUT USING 5-HYDROXYPROPANEONE AND PROPANEONE SPIKED 0.1 M ISOTONIC PHOSPHATE BUFFER.

NOTE: NO STATISTICALLY SIGNIFICANT (P > 0.05) DIFFERENCE BETWEEN THE NON-SPECIFIC LOSS OF 5-HYDROXYPROPANEONE TO DIALYSIS APPARATUS, AND MPS-1 ULTRAFLTRATION DEVICE AT DIFFERENT CONCENTRATIONS OF 5-HYDROXYPROPANEONE. KRUSKALL-WALLIS TEST.

The non-specific binding of PF and 5-OH-PF to MPS-1 ultrafiltration devices was conducted with PF and 5-OH-PF spiked 0.1 M phosphate buffer. Samples were spun in an ultracentrifuge at 2000 g for 20 minutes at 25 °C at a fixed angle of 18°. Both PF and 5-OH-PF showed high non-specific binding (Table 1) at both low and high concentrations of drug. The overall non-specific loss of 5-OH-PF and PF due to ultrafiltration were 27.5% and 21.5% respectively.
3.1.3 pH DEPENDENT BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE.

3.1.3.1 pH DEPENDENT BINDING OF PROPafenONE IN HUMAN SERUM, AND SOLUTIONS OF HUMAN SERUM ALBUMIN, AND HUMAN ALPHA-1-ACID GLYCOPROTEIN.

PF displayed pH dependent binding to serum, HSA (45 mg/mL), and AAG (0.9 mg/mL) dissolved in 0.1 M isotonic phosphate buffer. As the pH of the serum, HSA, and AAG increased, the free fraction of PF was decreased (e.g. increased binding)(Figure 3). Over a range of 0.6 pH units, the free fraction of PF in serum decreased by 50% (free fraction was 0.090 at pH 7.2 and 0.045 at pH 7.8). Similar findings of pH sensitive binding for PF were apparent for HSA, and AAG solutions (Figure 3).

3.1.3.2 pH DEPENDENT BINDING 5-HYDROXYPROPafenONE IN HUMAN SERUM, A SOLUTION OF HUMAN SERUM ALBUMIN, AND OF HUMAN ALPHA-1-ACID GLYCOPROTEIN.

The metabolite, 5-OH-PF, displayed pH dependent binding similar to that of the parent compound, PF (Figure 4). The free fraction of 5-OH-PF in serum and in a solution of AAG was decreased (increased binding) as the pH increased. Like the results observed for the parent compound, the free fraction of 5-OH-PF in serum decreases (increased binding) by 50% over a pH range of 0.6 pH units. That is, at a low pH of 7.2 the free fraction of 5-OH-PF in serum was 0.125 and at pH 7.8 the free fraction was 0.065. A similar trend in pH sensitive binding to HSA was not apparent.
Figure 3. The pH dependent binding of propafenone (2.0 µg/mL) in serum, and solutions of HSA and AAG.

Human AAG 0.90 mg/mL (○)
HSA 45.0 mg/mL (●)
Normal human serum (△)

(pH of protein side of dialysis cell following dialysis ± SD vs. free fraction ± SD).
Figure 4. The pH dependent binding of 5-hydroxypropafenone (0.5 μg/mL) in serum, and solutions of HSA and AAG.

Human AAG 0.90 mg/mL (○)
HSA 45 mg/mL (●)
Normal human serum (△)

(pH of protein side of dialysis cell following dialysis ± SD vs. free fraction ± SD).
3.1.3.3 The Influence of pH on the Partitioning of Propafenone and 5-Hydroxypropafenone in a Toluene:Phosphate Buffer and Hexane:Phosphate Buffer System.

The influence of pH on the partitioning of PF or 5-OH-PF from the aqueous layer into the organic layer of two organic/aqueous fluid systems (toluene:phosphate buffer and hexane:phosphate buffer) as a function of pH was assessed. Partitioning of PF from the aqueous layer in the hexane system was not observed; however, partitioning of PF from the aqueous layer of the toluene:phosphate buffer system was substantial (Figure 5). As pH of the buffer in the organic/aqueous fluid system was increased, the fraction of PF in the aqueous layer decreased significantly (Figure 5). This correlated well with the calculated ratio of ionized vs. non-ionized PF. These calculations were based on a known pKa value of 9.0 for PF. The partitioning of 5-OH-PF from the aqueous layer of the toluene:phosphate buffer system was substantial, as levels 5-OH-PF in the aqueous phase became undetectable after pH 7.6 (Figure 6). The partitioning of 5-OH-PF from the aqueous layer of the hexane:phosphate buffer system was less in comparison with the loss of 5-OH-PF from the aqueous layer of the toluene:phosphate buffer system (Figure 6).

3.1.4 The Effect of Buffer Strength on Binding of 5-Hydroxypropafenone.

The binding of 5-OH-PF in serum was assessed in the presence of a 0.067 M, 0.10 M, and 0.15 M pH 7.4 phosphate buffer (Figure 7). No statistically significant difference in the free
Figure 5. Influence of alterations in pH of the partitioning of propafenone (2.0 μg/mL) between aqueous and organic layers in a toluene:phosphate buffer system.

Fraction of propafenone remaining in aqueous layer ± SD vs. pH (○). Contralateral y-axis depicts the calculated ratio of ionized/nonionized propafenone vs. pH (■).
Figure 6. Influence of alterations in pH on the partitioning of 5-hydroxypropafenone (0.5 µg/mL) between aqueous and organic layers in various organic solvents.

toluene:phosphate buffer (○) system.
hexane:phosphate buffer (●) system.

Fraction of 5-hydroxypropafenone remaining in the aqueous layer ± SD vs. pH.
Figure 7. The effect of molar strength of phosphate buffer on the free fraction of 5-hydroxypropafenone in serum ± SD.
fraction between the three different buffer strengths was observed (Kruskall-Wallis Test; p > 0.05). The 0.10 M pH 7.4 isotonic phosphate buffer was used for all subsequent dialysis experiments.

3.1.5 DEGRADATION OF 5-HYDROXYPROPafenONE DURING EQUILIBRIUM DIALYSIS.

Experiments to assess the degree of chemical degradation of 5-OH-PF in serum and buffer during an eight hour dialysis period were conducted. Aliquots of serum and buffer were spiked with 5-OH-PF and incubated in the dialysis bath at 37 °C. Samples were removed hourly and the concentration of 5-OH-PF was determined. No degradation of 5-OH-PF occurred over the entire 8 hour time period required to reach binding equilibrium.

3.1.6 VOLUME SHIFTS

Following all equilibrium dialysis experiments, the volumes of the protein (serum) compartment and the buffer compartment were measured to assure that significant (≠ > 10%) [Lima et al., 1983] fluid shifts did not occur from one fluid compartment to another. It was observed that significant volume shifts ( > 10% ) did not occur during the eight hour dialysis time. However, volume shifts of approximately 5% were commonly encountered.
3.1.7 OSMOLARITY OF 5-OH-PF STOCK SOLUTIONS USED IN EQUILIBRIUM DIALYSIS STUDIES.

The osmolarity of the stock solutions of 5-OH-PF was assessed. Over the metabolite concentration range of 0.1 to 45.0 µg/mL no significant changes in osmolarity were observed. The osmolarity of the stock solutions was 267.3 ± 1.3 mOsm (Mean ± SD).

3.1.8 PROTEIN LOSS DURING EQUILIBRIUM DIALYSIS

Experiments were conducted to assess the reduction in the amount of protein due to decomposition, dilution, and leakage during eight hours of equilibrium dialysis. It was found that total protein concentration did not change significantly for serum, HSA solutions, and high density lipoproteins solutions; however, small changes were observed in the very low density lipoprotein solution (< 2.5%), and the low density lipoprotein solution (< 8.5%), as measured by a Lowry protein assay (Table 2). Further, it was shown that while no change in AAG occurred in serum, a loss of approximately 12% occurred when AAG was dialyzed alone (Table 2).

3.1.9 TEST FOR DIALYSIS MEMBRANE INTEGRITY FOLLOWING DIALYSIS

To assess the integrity of the dialysis membranes following each dialysis experiment, a small aliquot of the dialyzed buffer was added to 0.4 mL of 3% trichloroacetic acid (TCA). If precipitation was observed
TABLE 2. CHANGE IN TOTAL PROTEIN AND ALPHA-1-ACID GLYCOPROTEIN IN PLEXIGLASS DIALYSIS CELLS AND CELLULOSE DIALYSIS MEMBRANES DURING DIALYSIS.

<table>
<thead>
<tr>
<th></th>
<th>START</th>
<th>FINISH</th>
<th>% CHANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TOTAL PROTEIN BY LOWRY ASSAY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALBUMIN</td>
<td>39.7 mg/mL</td>
<td>39.9 ± 1.8 mg/mL</td>
<td>ND</td>
</tr>
<tr>
<td>SERUM</td>
<td>62.0 mg/mL</td>
<td>61.8 ± 2.7 mg/mL</td>
<td>ND</td>
</tr>
<tr>
<td>HDL (complex = 44-47% protein)</td>
<td>1.72 mg/mL</td>
<td>1.81 ± 0.06 mg/mL</td>
<td>5.0%</td>
</tr>
<tr>
<td>LDL (complex = 19-22% protein)</td>
<td>0.84 mg/mL</td>
<td>0.78 ± 0.02 mg/mL</td>
<td>-6.0%</td>
</tr>
<tr>
<td>VLDL (complex = 5-12% protein)</td>
<td>0.09 mg/mL</td>
<td>0.09 ± 0.01 mg/mL</td>
<td>ND</td>
</tr>
<tr>
<td><strong>ALPHA-1-ACID GLYCOPROTEIN BY RADIAL IMMUNODIFFUSION PLATES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERUM</td>
<td>0.62 mg/mL</td>
<td>0.63 ± 0.02 mg/mL</td>
<td>ND</td>
</tr>
<tr>
<td>ALPHA-1-ACID GLYCOPROTEIN</td>
<td>0.89 mg/mL</td>
<td>0.78 ± 0.06 mg/mL</td>
<td>-12.0%</td>
</tr>
</tbody>
</table>

*NOTE: DUE TO THE COEFFICIENT OF VARIATION, IF % CHANGE WAS BELOW 5% NO CHANGE COULD BE DETECTED.*

* SAMPLES WERE DIALYZED FOR EIGHT HOURS AT 37 °C.

following the TCA treatment, the samples were discarded; this occurred in less than 1% of the samples.

3.2 PROTEIN BINDING OF 5-HYDROXYPROPafenONE IN SERUM OF HEALTHY MALE VOLUNTEERS

Equilibrium dialysis binding experiments were conducted using serum individually collected from 5 healthy male volunteers. The data
were plotted according to the method of Rosenthal [bound/free vs. bound] (Figures 8a-e). The binding ratio (bound/free) at low concentrations in comparison to higher concentrations of 5-OH-PF was generally found to be highly variable (Figures 8a-e). Total protein and AAG concentrations for each of the volunteers are presented in Appendix 1. Analysis of the nontransformed data with the aid of the non-linear curve fitting program, ENZFITTER with robust weighting, resulted in 5 subjects fitting the 1 binding-site model (Table 3).

**TABLE 3: BINDING PARAMETERS OF 5-HYDROXYPROPAPAFENOINE IN HEALTHY HUMAN SERUM**

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>BINDING AFFINITY ((K_A) (M^{-1}) \pm SD)</th>
<th>BINDING CAPACITY ((NP) (M) \pm SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(1.18 \times 10^4 \pm 2.74 \times 10^1)</td>
<td>(2.88 \times 10^{-4} \pm 5.59 \times 10^{-7})</td>
</tr>
<tr>
<td>2</td>
<td>(7.97 \times 10^3 \pm 1.77 \times 10^3)</td>
<td>(6.10 \times 10^{-4} \pm 1.17 \times 10^{-4})</td>
</tr>
<tr>
<td>3</td>
<td>(1.43 \times 10^4 \pm 1.06 \times 10^1)</td>
<td>(3.10 \times 10^{-4} \pm 1.91 \times 10^{-7})</td>
</tr>
<tr>
<td>4</td>
<td>(3.43 \times 10^4 \pm 4.98 \times 10^3)</td>
<td>(1.81 \times 10^{-4} \pm 1.84 \times 10^{-5})</td>
</tr>
<tr>
<td>5</td>
<td>(1.78 \times 10^4 \pm 2.22 \times 10^3)</td>
<td>(3.43 \times 10^{-4} \pm 3.34 \times 10^{-5})</td>
</tr>
</tbody>
</table>

mean value \(1.72 \times 10^4 \pm 1.02 \times 10^4\) \(3.46 \times 10^{-4} \pm 1.59 \times 10^{-4}\)

The average free fraction of 5-OH-PF over a wide range of concentrations (0.12 - 35.3 \(\mu g/mL\)) was not statistically different; however, at the lowest concentration of 5-OH-PF (0.06 \(\mu g/mL\)) the free fraction of 5-OH-PF was statistically different from the free fraction at all other concentrations. A slight increase in free fraction was observed as the concentration of 5-OH-PF was increased; however, this
Figure 8. The relationship between the ratio of bound drug concentration/free drug concentration ± SD vs. bound drug concentration of 5-hydroxypropafenone in serum.

a) Rosenthal plot of subject 1.
b) Rosenthal plot of subject 2.
Figure 8. cont. The relationship between the ratio of bound drug concentration/free drug concentration ± SD vs. bound drug concentration of 5-hydroxypropafenone in serum.

c) Rosenthal plot of subject 3.
d) Rosenthal plot of subject 4.
Figure 8. cont. The relationship between the ratio of bound drug concentration/free drug concentration ± SD vs. bound drug concentration of 5-hydroxypropafenone in serum.

e) Rosenthal plot of subject 5.
did not reach statistical significance (table 4).

**TABLE 4. 5-HYDROXYPROPafenONE FREE FRACTION IN NORMAL SERA WITH INCREASING CONCENTRATIONS OF 5-HYDROXYPROPafenONE**

<table>
<thead>
<tr>
<th>TOTAL CONCENTRATION OF 5-HYDROXYPROPafenONE (µg/mL)</th>
<th>MEAN FREE FRACTION OF 5-HYDROXYPROPafenONE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.06 ± 0.01</td>
<td>0.094 ± 0.013**</td>
</tr>
<tr>
<td>0.12 ± 0.02</td>
<td>0.154 ± 0.038</td>
</tr>
<tr>
<td>0.28 ± 0.03</td>
<td>0.187 ± 0.036</td>
</tr>
<tr>
<td>0.55 ± 0.06</td>
<td>0.180 ± 0.016</td>
</tr>
<tr>
<td>3.52 ± 0.43</td>
<td>0.179 ± 0.044</td>
</tr>
<tr>
<td>8.01 ± 2.32</td>
<td>0.181 ± 0.035</td>
</tr>
<tr>
<td>17.26 ± 3.29</td>
<td>0.193 ± 0.011</td>
</tr>
<tr>
<td>35.30 ± 5.31</td>
<td>0.211 ± 0.034</td>
</tr>
</tbody>
</table>

** Statistically significant difference using Kruskall-Wallis test with p < 0.05

3.2.1 PROTEIN BINDING OF THE METABOLITE, 5-HYDROXYPROPafenONE, IN THE PRESENCE OF THE PARENT COMPOUND PROPafenONE AT A THERAPEUTIC CONCENTRATION AS DETERMINED BY EQUILIBRIUM DIALYSIS AND ULTRAFILTRATION.

The binding of the metabolite 5-OH-PF at a concentration of 0.5 µg/mL remained unaltered by the addition of a therapeutic concentration of 2.0 µg/mL of the parent compound, PF (table 5). The results obtained in the study of protein binding of PF and 5-OH-PF using ultrafiltration were consistently lower compared to the results obtained from equilibrium dialysis (Table 5).
TABLE 5

ASSESSMENT OF THE BINDING OF THE METABOLITE 5-
HYDROXYPROPafenONE IN VITRO BY EQUILIBRIUM DIALYSIS AND ULTRAFILTRATION
WITH AND WITHOUT THE ADDITION OF THERAPEUTIC CONCENTRATIONS OF THE
PARENT COMPOUND PROPafenONE.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>FREE FRACTION EQUILIBRIUM DIALYSIS ± SD</th>
<th>FREE FRACTION ULTRAFILTRATION ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HYDROXYPROPafenONE ALONE (0.5 µg/mL)</td>
<td>0.223 ± 0.015</td>
<td>0.160 ± 0.003</td>
</tr>
<tr>
<td>5-HYDROXYPROPafenONE (0.5 µg/mL) WITH PROPafenONE (2.0 µg/mL)</td>
<td>0.232 ± 0.020</td>
<td>0.154 ± 0.008</td>
</tr>
<tr>
<td>PROPafenONE (2.0 µg/mL) WITH 5-HYDROXY-PROPafenONE (0.5 µg/mL)</td>
<td>0.063 ± 0.004</td>
<td>0.019 ± 0.003</td>
</tr>
</tbody>
</table>

3.3 BINDING PROFILES OF PROPafenONE AND 5-HYDROXYPROPafenONE TO ISOLATED HUMAN SERUM PROTEINS, LIPOPROTEIN DEFICIENT SERUM, AND NORMAL SERUM.

3.3.1 BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE TO ISOLATED HUMAN SERUM PROTEINS.

The differences in the extent of binding of PF and 5-OH-PF to isolated human serum proteins in a system containing therapeutic concentrations of both PF and 5-5-hydroxypropafenone was assessed. Large differences in the free fraction of PF and 5-OH-PF were observed in solutions of AAG dissolved in buffer, AAG plus HSA dissolved in buffer, serum, and lipoprotein deficient serum (Figure 9). A smaller difference was observed between the binding of PF and 5-OH-PF in a solution of HSA dissolved in buffer (Figure 9).
Figure 9. Comparison of binding of propafenone (2.0 μg/mL) and 5-hydroxypropafenone (0.5 μg/mL) in AAG, HSA, HSA + AAG, serum and lipoprotein deficient serum.

HSA (39.0 mg/mL) in 0.1 M phosphate pH 7.51 ± 0.01 buffer.
Human AAG (0.71 mg/mL) in 0.1 M phosphate pH 7.55 ± 0.05 buffer.
HSA (39.0 mg/mL) and Human AAG (0.68 mg/mL) in 0.1 M phosphate pH 7.50 ± 0.01 buffer.
Serum (47.0 mg/mL total protein) pH 7.40 ± 0.05.
Lipoprotein deficient serum (36.5 mg/mL total protein) pH 7.52 ± 0.03.

(Free fraction ± SD).
3.3.2 BINDING OF PROPAFENONE AND 5-HYDROXYPROPafenone TO PURE ISOLATED HUMAN SERUM PROTEINS DIssOLVED IN EITHER BUFFER OR SERUM ULTRAFILTRATE.

Pure human serum proteins (HSA, AAG, and HSA plus AAG) were dissolved in either buffer or serum ultrafiltrate (for the composition of serum ultrafiltrate see Appendix 3). The binding (free fraction) of PF and 5-OH-PF to these proteins was then assessed. The difference in binding of PF to AAG in buffer vs. serum ultrafiltrate did reach statistical significance [two sample t-test p < 0.05] (Figure 10). Further, no difference in pH between the buffer and serum ultrafiltrate, which could account for the observed binding difference of PF in AAG, was observed. No statistical difference was observed between PF binding to HSA dissolved in buffer and serum ultrafiltrate (Figure 10). However, a statistical difference was apparent [two sample t-test p < 0.05] between buffer and ultrafiltrate when both AAG and HSA were dissolved together (Figure 10). The difference in pH between the AAG together with HSA in either buffer or ultrafiltrate also reached statistical significance [two sample t-test p < 0.05].

The use of buffer or ultrafiltrate to dissolve the pure isolated human serum proteins did not result in any significant differences in the binding of 5-OH-PF [two sample t-test p=0.05] to AAG, HSA, and HSA together with AAG (Figure 11).
Figure 10. The effect of buffer vs. human serum ultrafiltrate as solvents to dissolve AAG, HSA, and AAG + HSA on the free fraction (± SD) of propafenone (2.0 μg/mL).

Human AAG (0.71 mg/mL) in 0.1 M phosphate pH 7.50 ± 0.01 buffer, and (0.67 mg/mL) in human serum ultrafiltrate pH 7.48 ± 0.08.

HSA (39.0 mg/mL) in 0.1 M phosphate pH 7.51 ± 0.01 buffer, and (40.0 mg/mL) in human serum ultrafiltrate pH 7.40 ± 0.07

HSA (39.0 mg/mL) + human AAG (0.68 mg/mL) in 0.1 M phosphate pH 7.50 ± 0.01 buffer, and 39.1 mg/mL and 0.71 mg/mL, respectively, in human serum ultrafiltrate pH 7.32 ± 0.04.

* Statistically significant difference; two sample t-test with p=0.05 between treatment groups of same drug.
Figure 11. The effect of buffer vs. human serum ultrafiltrate as solvents to dissolve AAG, HSA, and AAG + HSA on the free fraction (± SD) of 5-hydroxy-propafenone (0.5 µg/mL).

Human AAG (0.71 mg/mL) in 0.1 M phosphate pH 7.50 ± 0.01 buffer, and (0.67 mg/mL) in human serum ultrafiltrate pH 7.48 ± 0.08.

HSA (39.0 mg/mL) in 0.1 M phosphate pH 7.51 ± 0.01 buffer, and (40.0 mg/mL) in human serum ultrafiltrate pH 7.40 ± 0.07.

HSA (39.0 mg/mL) + human AAG (0.68 mg/mL) in 0.1 M phosphate pH 7.50 ± 0.01 buffer, and 39.1 mg/mL and 0.71 mg/mL, respectively, in human serum ultrafiltrate pH 7.32 ± 0.04.

* Statistically significant difference; two t-test with p=0.05 between treatment groups of same drug.
3.3.3 THE BINDING OF PROPAFENONE AND 5-HYDROXYPROPAFENONE TO FREE FATTY ACID FREE HSA COMPARED TO NORMAL ALBUMIN.

The binding (free fraction) of PF and 5-OH-PF to free fatty acid free HSA and HSA with endogenous free fatty acids was assessed. The difference in the free fractions of PF and 5-OH-PF between free fatty acid free HSA and HSA was statistically significance [two sample t-test p=0.05]. The free fraction of both PF and 5-OH-PF decreased in the free fatty acid HSA group compared to the normal HSA group. It should be noted that the pH difference between the free fatty acid free HSA and HSA could account for the observed statistical difference in the free fraction of PF and 5-OH-PF in these treatment groups (Figure 12). The pH in the HSA group was 7.51 ± 0.03 vs. 7.60 ± 0.05 in the free fatty acid free HSA group.

3.3.4 BINDING OF PROPAFENONE AND 5-HYDROXYPROPAFENONE TO LIPOPROTEIN DEFICIENT SERUM vs. NORMAL SERUM

Differences in binding due to the presence or absence of human lipoproteins in serum were assessed. There was no statistically significant difference [two sample t-test p > 0.05] between the free fraction of PF in lipoprotein deficient serum vs. normal serum (Figure 13). However, a statistically significant difference was apparent between the free fraction of 5-OH-PF in lipoprotein deficient serum vs. normal serum [two sample t-test p < 0.05] (Figure 13).
Figure 12. Comparison between the free fraction (± SD) of propafenone (2.0 μg/mL) + 5-hydroxypropafenone (0.5 μg/mL) in free fatty acid free albumin, and in HSA.

HSA (39.0 mg/mL) in 0.1 M phosphate pH 7.51 ± 0.01 buffer.
Free fatty acid free HSA (35.8 mg/mL) in 0.1 M phosphate pH 7.60 ± 0.02 buffer.

* Statistically significant difference; two t-test with p=0.05 between treatment groups of same drug.
Figure 13. Comparison of the free fraction (± SD) of propafenone (2.0 μg/mL) and 5-hydroxypropafenone (0.5 μg/mL) in lipoprotein deficient serum, and normal serum.

Serum: Total protein 46.8 mg/mL, AAG 0.69 mg/mL pH 7.40 ± 0.05.
Lipoprotein deficient serum: Total protein 36.5 mg/mL, AAG 0.90 mg/mL pH 7.52.

* Statistically significant difference; two t-test with p=0.05 between treatment groups of the same drug.
3.3.5 DISPLACEMENT OF PROPafenONE AND 5-HYDROXYPROPafenONE FROM PROTEIN BINDING SITES BY IBUPROFEN AND DISOPYRAMIDE.

The effects of the addition of drugs capable of displacing other drugs from serum protein binding sites were evaluated for the binding of PF and 5-OH-PF in serum. Ibuprofen, a non-steroidal anti-inflammatory drug commonly used as a probe to displace acid drugs from HSA, was found to increase the free fraction of PF in serum by 37% and 35% compared to control at concentrations of 2.0 and 50.0 μg/mL respectively (Table 6). Further, ibuprofen was also found to increase the free fraction of 5-OH-PF in serum by 21% and 27% compared to control at concentrations of 2.0 and 50.0 μg/mL respectively (Table 6).

TABLE 6. DISPLACEMENT OF PROPafenONE AND 5-HYDROXYPROPafenONE FROM HUMAN SERUM PROTEIN BINDING SITES BY DISOPYRAMIDE AND IBUPROFEN

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>MEAN FREE FRACTION OF 5-HYDROXY-PROPafenONE (± SD)</th>
<th>MEAN FREE FRACTION OF PROPafenONE (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td>0.215 ± 0.005</td>
<td>0.063 ± 0.004</td>
</tr>
<tr>
<td>IBUPROFEN 2.0 μg/mL</td>
<td>0.260 ± 0.016*</td>
<td>0.086 ± 0.013*</td>
</tr>
<tr>
<td>IBUPROFEN 50.0 μg/mL</td>
<td>0.273 ± 0.010*</td>
<td>0.084 ± 0.003*</td>
</tr>
<tr>
<td>DISOPYRAMIDE 2.0 μg/mL</td>
<td>0.275 ± 0.013*</td>
<td>0.110 ± 0.006*</td>
</tr>
<tr>
<td>DISOPYRAMIDE 8.0 μg/mL</td>
<td>0.265 ± 0.033*</td>
<td>0.133 ± 0.008*</td>
</tr>
</tbody>
</table>

* Statistically significant from control using a Kruskall-Wallis test with p < 0.05

Disopyramide, is an amine antiarrhythmic drug known to bind specifically with high affinity to AAG [Kremer et al., 1988], thus
Disopyramide was used as a probe for displacing basic drugs such as PF and 5-OH-PF from specific AAG binding sites. At concentrations of 2.0 and 8.0 µg/mL, disopyramide was found to increase the free fraction of both 5-OH-PF and PF. The increase in free fraction of 5-OH-PF was 28% and 23% of control at concentrations of 2.0 and 8.0 µg/mL of disopyramide respectively. The increase in the free fraction of PF was significantly larger than 5-OH-PF (74% and 111% of control at 2.0 and 8.0 µg/mL of disopyramide respectively).

3.3.6 DISPLACEMENT OF PROPafenONE AND 5-HYDROXYPROPafenONE FROM ISOLATED HUMAN SERUM PROTEINS BY DISOPYRAMIDE AND IBUPROFEN

The influence of the addition of the binding displacers, such as ibuprofen and disopyramide, to isolated proteins was assessed. Statistically significant differences in the free fraction of PF were observed in all groups (HSA, AAG) treated with the displacing agent disopyramide in comparison to control (Figure 15) [two sample t-test p < 0.05]. Notable differences in pH which could alter binding were observed only in the HSA group. The addition of 50 µg/mL of ibuprofen also resulted in statistically significant [two sample t-test p=0.05] differences in the free fraction of PF when compared to control (Figure 14).

Binding of 5-OH-PF in HSA and AAG was altered by the addition of disopyramide only in the HSA group [two sample t-test p < 0.05] (Figure 15). There was no apparent differences between the binding of 5-OH-PF with the addition of disopyramide in the AAG test group. A pH difference in the HSA was observed between the control and
Figure 14. The effect of the addition of ibuprofen (50.0 μg/ml) on the free fraction (± SD) of propafenone (2.0 μg/ml) and 5-hydroxypropafenone (0.5 μg/ml)

Serum: Total protein 46.5 mg/mL, AAG 0.69 mg/mL pH (test) 7.43 and (control) 7.40.
HSA: Total protein 39.0 mg/mL in 0.1 M phosphate pH (test) 7.51 and (control) 7.50 buffer.
AAG: AAG 0.71 mg/mL in 0.1 M phosphate pH (test) 7.50 and (control) 7.55 buffer.

* Statistically significant difference; two t-test with p=0.05 between treatment groups of the same drug.

** Statistically significant difference; Mann Whitney U-test with p=0.05 between treatment groups of the same drug.
Figure 15. The effect of the addition of disopyramide (8.0 μg/mL) on the free fraction ± SD of propafenone (2.0 μg/mL) and 5-hydroxypropafenone (0.5 μg/mL).

Serum: Total protein 46.5 mg/mL, AAG 0.69 mg/mL pH (test) 7.40 and (control) 7.41.
HSA: Total protein 39.0 mg/mL in 0.1 M phosphate pH (test) 7.60 and (control) 7.50 buffer.
AAG: AAG 0.71 mg/mL in 0.1 M phosphate pH (test) 7.50 and (control) 7.55 buffer.

* Statistically significant difference; two t-test with p=0.05 between treatment groups of the same drug.

** Statistically significant difference; Mann Whitney U-test with p=0.05 between treatment groups of the same drug.
disopyramide group. The addition of ibuprofen altered the free fraction of 5-OH-PF only in the HSA group [two sample t-test p < 0.05] (Figure 14). The addition of 50 μg/mL of ibuprofen did not result in any significant difference in the binding of 5-OH-PF to AAG [two sample t-test p > 0.05].

3.3.7 PURITY ASSURANCE OF ISOLATED PROTEIN IN BINDING EXPERIMENTS

The composition and concentrations of proteins in each of the protein solutions used in the above experiments was determined and is presented in Appendix 2. The composition of the ultrafiltrate solution is compared to normal serum in Appendix 3. Finally, the SDS PAGE separation studies to assure purity results of the isolated protein binding solutions used in the mentioned binding experiments are presented in Appendix 4.

3.4 BINDING CHARACTERISTICS OF PROPAFENONE AND 5-HYDROXYPROPAFENONE.

3.4.1 BINDING CHARACTERISTICS OF PROPAFENONE AND 5-HYDROXYPROPAFENONE IN ALPHA-1-ACID GLYCOPROTEIN SOLUTION.

The binding interaction of PF and 5-OH-PF with AAG was qualitatively evaluated using a Rosenthal plot. Binding of PF and 5-OH-PF was measured over a wide range of drug concentrations, and the binding ratio (bound/free) was plotted against the concentration of bound drug (Figure 16a and 16b). Curve fitting was done using the
Figure 16. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in a physiological concentration of AAG (19.3 μM) in 0.1 phosphate pH 7.40 buffer.

Rosenthal plot of propafenone (○)
Rosenthal plot of 5-hydroxypropafenone (▲)
program ENZFITTERR of the non-transformed data [Leatherbarrow, 1987]. PF appears to have two distinct types of binding sites on AAG; one high affinity, low capacity ($K_a 1.1 \times 10^5$ M$^{-1}$, $N_P 5.7 \times 10^{-1}$), and one low affinity high capacity ($K_a 6.6 \times 10^{-4}$ M, $N_P 3.40 \times 10^1$), whereas 5-OH-PF only shows one distinct type of binding site ($K_a 3.20 \times 10^2$ M$^{-1}$, $N_P 1.1 \times 10^5$ M, $N_P 5.7 \times 10^1$) on AAG. PF binding to AAG shows concentration-dependent binding at low concentrations, followed by a sharp rise in the free fraction at high drug concentrations (Figure 22). Although 5-OH-PF shows a similar sharp rise in the free fraction at high concentrations of drug, there is little evidence to suggest that 5-OH-PF displays concentration dependent binding to AAG at lower concentrations similar to that observed for PF (Figure 23).

3.4.2 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN HUMAN SERUM ALBUMIN SOLUTION

Both PF and 5-OH-PF tend to bind to a lesser degree in human serum HSA solution as evidenced by their respective Rosenthal plots (Figures 17a and 17b). Following non-linear regression with ENZFITTERR of the non-transformed data [Leatherbarrow, 1987], the 1 specific site model displayed the best fit with the best random distribution of y-axis residuals. The binding of both PF and 5-OH-PF to HSA was low affinity and high capacity (PF - $K_a 2.94 \times 10^6$ M$^{-1}$, $N_P 1.1 \times 10^{-5}$ M, $N_P 5.7 \times 10^{-1}$; 5-OH-PF - $K_a 2.1 \times 10^2$ M$^{-1}$, $N_P 7.8 \times 10^{-3}$ M, $N_P 1.24 \times 10^1$). PF and 5-OH-PF also do not show concentration dependent binding to HSA as would be evidenced by steadily increasing free fraction as the concentration
Figure 17. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in a physiological concentration of HSA (630.0 μM) in 0.1 phosphate pH 7.36 buffer.

Rosenthal plot of propafenone (▼)
Rosenthal plot of 5-hydroxypropafenone (●)
Figure 22. Free fraction ± SD of propafenone at various concentrations in solutions of human serum proteins.

HSA (630 μM) 40.9 mg/mL (△) pH 7.36.
Human AAG (19.3 μM) 0.88 mg/mL (○) pH 7.40.
Serum (total protein 48.9 mg/mL, Human AAG 0.79 mg/mL) (○) pH 7.47.
HDL complex (15.3 μM) 3.8 mg/mL (▼) pH 7.32.
LDL complex (1.8 μM) 4.2 mg/mL (●) pH 7.32.
Vldl complex (0.17 μM) 1.2 mg/mL (◆) pH 7.32.
Figure 23. Free fraction ± SD of 5-hydroxypropafenone at various concentrations in solutions of human serum proteins.

HSA (630 μM) 40.9 mg/mL (△) pH 7.36.
Human AAG (19.3 μM) 0.88 mg/mL (●) pH 7.40.
Serum (total protein 48.9 mg/mL, Human AAG 0.79 mg/mL) (○) pH 7.47.
HDL complex (15.3 μM) 3.8 mg/mL (□) pH 7.32.
LDL complex (1.8 μM) 4.2 mg/mL (▲) pH 7.32.
Vldl complex (0.17 μM) 1.2 mg/mL (■) pH 7.32.
increases (Figures 22 and 23)

3.4.3 BINDING CHARACTERISTICS OF PROPafenone AND 5-HYDROXYPROPafENONE IN POOLED HUMAN SERUM.

The Rosenthal plot of PF indicated that two distinct types of binding sites were present in pooled human serum (Figure 18a). Following curve fitting (ENZFITTER\textsuperscript{R}) of the non-transformed data two distinct binding sites were apparent ($K_a_1$ 9.16 X $10^4$ M\textsuperscript{-1}, $N_P\_1$ 9.51 X $10^{-5}$ M, $K_a_2$ 2.31 X $10^3$ M\textsuperscript{-1}, $N_P\_2$ 1.66 X $10^{-3}$ M) for PF in serum. While the Rosenthal plot of 5-OH-PF binding in pooled serum shows some scatter (Figure 18b), following curve fitting 1 binding site was apparent ($K_a$ 2.06 X $10^4$ M\textsuperscript{-1}, $N_P$ 2.20 X $10^{-4}$ M). Concentration dependent binding of PF in pooled human serum, was observed particularly at higher concentrations (Figure 22). The metabolite, 5-OH-PF, does not seem to undergo concentration dependent binding, as evidenced by the relatively constant free fraction over the concentrations examined (Figure 23).

3.4.4 BINDING CHARACTERISTICS OF PF AND 5-HYDROXYPROPafENONE IN A SOLUTION OF HIGH DENSITY LIPOPROTEINS.

The Rosenthal plot of PF binding to high density lipoproteins shows the possible presence of two distinct types of binding sites (Figure 19a). However, following curve fitting of the non-transformed data (ENZFITTER\textsuperscript{R}) only one specific saturable site was apparent ($K_a$ 1.1 X $10^4$ M\textsuperscript{-1}, $N_P$ 2.6 X $10^{-4}$ M, $N$ 1.69 X $10^1$). Over a wide concentration range, PF (in a solution of high density lipoproteins)
Figure 18. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in serum.

Serum total protein 49.0 mg/mL, AAG 0.79 mg/mL (19.3 µM) pH 7.47.

Rosenthal plot of propafenone (△)
Rosenthal plot of 5-hydroxypropafenone (▲)
Figure 19. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in a physiological concentration of HDL (15.3 μM complex, 1.72 mg/mL total protein) in 0.1 phosphate pH 7.32 buffer.

Rosenthal plot of propafenone (▼)
Rosenthal plot of 5-hydroxypropafenone (○)
tends to show a gradual rise in free fraction which would suggest concentration-dependent binding (Figure 22). The Rosenthal plot of 5-OH-PF in the HDL solution shows data scatter; however, only one type of binding site seems apparent. Following curve fitting only non-specific binding site was apparent ($K_{\text{non-specific}} 3.08$). When the affinity of the binding site is very small, the equation for the one site binding model collapses such that the (non-specific) binding is the product of the affinity constant and the binding capacity (see Equation 11 Section 2.8.2 [Glasson et al. 1980]). There is no concentration-dependent binding of 5-OH-PF to high density lipoproteins (Figure 23).

3.4.5 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN A SOLUTION OF LOW DENSITY LIPOPROTEINS.

The Rosenthal plot of both propafenone and 5-OH-PF in a solution of LDL tends to suggest the presence of only one type of binding site (Figure 20a and 20b). In fact, only non-specific binding was apparent for both PF ($K_{\text{non-specific}} 13.9$) and 5-OH-PF ($K_{\text{non-specific}} 12.3$) after modeling and curve fitting. The free fraction of both PF and 5-OH-PF over a wide range of concentrations in a solution of low density lipoproteins is very low, and does not change with increasing concentrations.

3.4.6 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN A SOLUTION OF VERY LOW DENSITY LIPOPROTEINS.

The binding of PF to very low density lipoproteins was
Figure 20. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of PF and 5-hydroxypropafenone in a physiological concentration of LDL (1.8 μM complex, 0.84 mg/mL total protein) in 0.1 phosphate pH 7.32 buffer.

Rosenthal plot of propafenone (◊)
Rosenthal plot of 5-hydroxypropafenone (▲)
very low, and thus resulted in a poor spread of data on the x-axis of the Rosenthal plot (Figure 21a). Although the binding 5-OH-PF was also very low, it was not as low as PF binding to very low density lipoproteins (Figure 21b). Both drugs displayed low non-specific binding (PF - $K_{\text{non-specific}}$ 0.065, 5-OH-PF - $K_{\text{non-specific}}$ 0.593). The free fraction of PF was very high and non-uniform (Figure 22). While the free fraction of 5-OH-PF over a wide concentration range was also very high (approximating 1), it showed significantly less scatter (Figure 23).

3.4.7 MATHEMATICAL BINDING EQUATIONS FOR PROPAFENONE AND 5-HYDROXYPROPafenONE.

Equations describing the best fit of PF and 5-OH-PF binding to individual proteins were summed to arrive at equations that would attempt to approximate the binding of both PF and 5-OH-PF in serum.

$$PF \text{ bound} = \frac{[(0.011)(2943)(DF) + (0.32)(0.66)(DF)]^{aag} + (0.065)(DF)}{(1+2943(DF))} + \frac{(0.32)(DF)}{(1+0.32(DF))}$$
$$+ \frac{[(4.72)(0.06)(DF) + 0.693(DF)]^{alb} + (1+4.72(DF))}{(1+11.02(DF))}$$
$$+ \frac{[(11.02)(0.26)(DF)]^{hDL} + (1+11.02(DF))}{(1+11.02(DF))}$$
$$+ [13.9(DF)]^{LDL} + [0.07(DF)]^{VLDL}$$
Figure 21. Relationship between the ratio of bound drug concentration/free drug concentration vs. bound drug concentration of propafenone and 5-hydroxypropafenone in a physiological concentration of VLDL (0.17 μM complex, 0.09 mg/mL total protein) in 0.1 phosphate pH 7.32 buffer.

Rosenthal plot of propafenone (◇)
Rosenthal plot of 5-hydroxypropafenone (▼)
5-OH-PF bound (mM) = \frac{[(313)(0.0094)(DF)]^{aag} +}{(1+313(DF))} \frac{[(0.21)(7.83)(DF)]^{alb} +}{(1+0.21(DF))} \frac{[3.08(DF)]^{hdl} + [12.3(DF)]^{ldl} + [0.59(DF)]^{vldl}}{}

where DF represents the free PF or 5-OH-PF in mMolar. The binding affinity and capacity constants were estimated from binding of PF and 5-OH-PF in solutions of isolated serum proteins (AAG, HSA, HDL, LDL, and VLDL) in the previous sections. The amount of bound PF and 5-OH-PF was calculated and compared to the actual data points Figures 23 and 24, respectively. Stepwise the binding contribution of individual proteins was removed (ie. PF bound - contribution of very low density lipoproteins followed by PF bound - contribution of VLDL and LDL etc. until the contribution of only AAG remained) and the amount of bound PF and 5-OH-PF calculated and plotted (Figures 24 and 25, respectively). The binding calculated with the mathematical reconstitution equation (composed of the sums of individual binding contributions) was consistently high for both PF and 5-OH-PF when compared to the actual data points. Upon the removal of both VLDL and LDL, the calculated binding more closely resembled the actual binding of PF and 5-OH-PF (Figures 24 and 25).
Figure 24. A direct plot of propafenone binding (bound vs. free) calculated by mathematically reconstructing binding contributions of serum protein constituents and subsequent removal of binding contributions of individual serum proteins.

AAG + HSA + HDL + LDL + VLDL (▲)
AAG + HSA + HDL + LDL - VLDL (▲)
AAG + HSA + HDL - LDL - VLDL (▲)
AAG + HSA - HDL - LDL - VLDL (▲)
AAG - HSA - HDL - LDL - VLDL (▼)
CONTROL SERUM DATA (○)
Figure 25. A direct plot of 5-hydroxypropafenone binding (bound vs. free) calculated by mathematically reconstructing binding contributions of serum protein constituents and subsequent removal of binding contributions of individual serum proteins.

AAG + HSA + HDL + LDL + VLDL (△)
AAG + HSA + HDL + LDL - VLDL (▲)
AAG + HSA + HDL - LDL - VLDL (□)
AAG + HSA - HDL - LDL - VLDL (◇)
AAG - HSA - HDL - LDL - VLDL (▼)
CONTROL SERUM DATA (○)
3.4.8 PROTEIN QUALITY ASSURANCE DURING BINDING CHARACTERIZATION EXPERIMENTS

Total protein concentrations, and the concentration of AAG used in the binding characterization experiments are presented in Appendix 5. Results from the agarose electrophoresis separation before and after dialysis of the lipoproteins suggest that the lipoprotein complex remains intact during dialysis (Appendix 6).

3.5 UPTAKE OF PROPAFENONE AND 5-HYDROXYPROPAFENONE INTO RED BLOOD CELLS

3.5.1 TIME TO EQUILIBRIUM FOR RED BLOOD CELL UPTAKE OF PROPAFENONE AND 5-HYDROXYPROPAFENONE.

Initial experiments to determine the time course of red blood cell uptake of PF and 5-OH-PF were conducted. The uptake equilibrium for PF and 5-OH-PF was very rapid. Samples taken within 5 minutes after the start of incubation showed uptake equilibrium was attained almost immediately, since the concentration in the red blood cells had not changed for the entire 35 minute incubation time. An incubation time of 30 minutes was chosen to conduct all red blood cell uptake experiments.

3.5.2 UPTAKE OF PROPAFENONE AND 5-HYDROXYPROPAFENONE BY RED BLOOD CELLS

The uptake ratio (concentration of drug in red blood
cells/concentration of drug in supernatant) of both PF and 5-OH-PF in red blood cells was substantial when buffer was used as the supernatant (Figure 26). The uptake ratio of 5-OH-PF was almost double that of PF in the buffer supernatant system. This observation did reach statistical significance [two sample t-test p ≥ 0.05].

The uptake ratio in red blood cell : serum supernatant system of PF and 5-OH-PF was substantially less than that observed for the buffer system (Figure 26). The uptake ratio of 5-OH-PF was almost six fold greater that PF [two sample t-test p < 0.05].

The uptake ratio of PF and 5-OH-PF in a whole blood system showed similar results when compared to the system in which serum was used as the supernatent (Figure 26). The uptake ratio of 5-OH-PF was almost 5 fold greater than the uptake ratio of PF [two sample t-test p < 0.05].
Figure 26. Comparison of the ratio (drug concentration in RBC/drug concentration in supernatant) of propafenone and 5-hydroxypropafenone with buffer, serum, and plasma as supernatants.

RBC 45% v/v suspension
* Statistically significant difference; two t-test with p ≤ 0.05 between treatment groups of the same drug.
4. DISCUSSION

4.1 PRELIMINARY BINDING EXPERIMENTS

4.1.1 PROTEIN BINDING AND PROPafenone

PF is a type IC antiarrhythmic agent which as been found to be useful in the treatment of a number of different arrhythmias [Schlepper, 1987]. As with most antiarrhythmic agents, PF is believed to display a steep-dose response curve [Connolly et al., 1983b; Siddoway et al., 1984], suggesting that small alterations in serum drug concentration may have disproportionate pharmacological effects. Further, since it is generally accepted that the free drug concentration better correlates to the observed pharmacological effect (free drug distributes to the site of action) [Svensson et al., 1986], factors affecting free drug concentration of drugs must be carefully characterized.

PF is a high clearance drug [Holloman et al., 1984; Axelson et al., 1987]; therefore, alterations in binding, provided that the volume of distribution remains unaltered, would not be expected to alter pharmacokinetic parameters of total drug (e.g. clearance of a high clearance drug is not dependent on free drug concentration). However, depending on the direction of the drug-protein binding interaction (e.g. increase or decrease) the concentration of free drug would be altered [Svensson et al., 1986]. Since PF is highly bound at therapeutic concentrations to AAG in serum [Gilles et al., 1985; Chan et al., 1989b], small alterations in binding may result in very large changes in the free fraction of the drug, and therefore, effect. Thus, it is very
important to accurately characterize the binding of PF and to be able to predict the effect of altered binding on free drug concentration, and further pharmacological effect.

The metabolite, 5-OH-PF, has been found to have similar activity to the parent compound (e.g. class 1C antiarrhythmic properties) [Malfatto et al., 1988; Rouet et al., 1989]. Since, 5-OH-PF is believed to contribute to the pharmacological effect observed following systemic administration of PF [Kates et al., 1985], factors affecting the free drug concentration of 5-hydroxypropafone must be carefully studied. Further, no efforts have yet been made to carefully characterize the binding of PF and 5-OH-PF, and to determine proteins which account for the majority of binding at therapeutic concentrations. Therefore, it is the aim of this study to consider factors affecting the binding of both PF and its active metabolite, 5-OH-PF.

4.1.2 EQUILIBRIUM DIALYSIS: TIME TO EQUILIBRIUM

Many techniques are now available for the separation of free and protein bound drug; however, ultrafiltration and equilibrium dialysis are most commonly employed due to the ease of use and proven reliability [Kwong, 1985]. Ultrafiltration, a rapid method for measuring drug-protein binding, like equilibrium dialysis has several disadvantages, such as, temperature control, limited capacity to run large numbers of samples, and non-specific binding [Kwong, 1985]. However, ultrafiltration can provide reliable estimates of drug-protein binding if the method is rigorously validated and controlled [Kwong, 1985].
Equilibrium dialysis, despite its potential disadvantages, such as Donnan effects [Lindup, 1987], volume shifts [Lima et al., 1983], non-specific binding [Kwong, 1985], and pH shifts during dialysis [Lui and Chiou, 1986], still remains the standard method for evaluating drug-protein binding. Equilibrium dialysis can provide reproducible estimates of drug-protein binding if the experimental conditions are optimized, and steps taken to correct for the above mentioned problems [Kristensen and Gram, 1981].

Initially in equilibrium dialysis studies, the time required to reach drug-protein binding equilibrium must be optimized. The time to attain equilibrium is dependent on many factors, namely, the surface to volume ratio of the dialysis system, the pore size of the membrane [Lindup, 1987], and whether the drug is added to the serum or buffer compartment of the dialysis cell [McNamara and Bogardu, 1982]. It is important to optimize the dialysis time in order to reduce or avoid problems, such as pH shift, volume shifts, and bacterial growth [Kwong, 1985]; however, if not enough time to reach equilibrium is provided the variance of the binding measurement will be greater (figure 2b), and drug-protein binding measurements may be incorrect. The time required to reach equilibrium for PF was found to be 6 hours [Chan et al., 1989b], while the metabolite, 5-OH-PF, reached equilibrium in 8 hours (figure 2a & 2b). These may be considered intermediate times, since equilibrium dialysis times have been reported to range from less than 4 hours [Lindup, 1987] to as high as 16 hours [Tozer 1981]. Fortunately, short and intermediate dialysis times reduce the degree of time dependent problems such as, volume shifts [Tozer et al., 1981], pH
shifts [Lui and Choiu, 1986], and bacterial growth [Kwong, 1985]

4.1.3 NON-SPECIFIC BINDING OF PF AND 5-HYDROXYPROPafenONE

Since both PF [Chan et al., 1989b] and 5-OH-PF (Table 1) showed high and variable non-specific binding with equilibrium dialysis and ultrafiltration, the use of equilibrium dialysis to measure drug-protein binding would be considered advantageous over ultrafiltration. This is so because a correction factor to account for the drug lost to the ultrafiltration devices is required before drug protein binding can be calculated. Thus, an additional error due to the variability of the non-specific binding estimate is introduced to the measurement of drug-protein binding. The same is not true for equilibrium dialysis, since the binding calculation (free fraction) determined using the post-dialysis serum and buffer drug concentration automatically accounts for the non-specific binding. That is, the drug concentration measured in the serum compartment represents both the protein bound and the free drug in serum. At equilibrium, the buffer drug concentration will represent the free drug concentration in the serum side of the dialysis cell. In both of these measurements, since non-specifically bound drug is not included, only the drug protein binding independent of the non-specific binding will be measured [Kwong, 1985]. This would still be true in the situation where non-specific binding is unequal between the buffer and the serum side of the dialysis cell, unless the drug which is non-specifically bound could alter the equilibrium of the free drug, which is unlikely.
It should be noted that the total drug concentration is not equal to the concentration of drug initially added to the dialysis cell. Calculations of drug-protein binding made using the concentration of drug added to the dialysis cell would fail to account for both the amount of drug which is non-specifically bound, and the amount of drug which has diffused to the buffer compartment of the dialysis cell. These calculations would incorrectly overestimate the binding [Kwong, 1985].

4.1.4 pH DEPENDENT BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE

Differences in drug-protein binding related to pH have been observed for many drugs, including oxprenolol and betaxolol [Henry and Mitchell, 1980], tolmetin [Matsuyama et al., 1987], theophylline and quinidine [Bors et al., 1984]. In the present study, PF protein binding in serum increased proportionally to increases in pH over the range studied (pH 7.0 -8.0)(Figure 3). Similar pH dependent alterations in drug-protein binding were apparent in solutions of AAG and HSA (Figure 3). The metabolite, 5-OH-PF, displays similar binding patterns in serum and in a solution of AAG (Figure 4), while drug-protein binding to HSA did not show pH dependence; reasons for this unexpected behaviour were not apparent.

The observed pH dependent drug-protein binding could result from pH induced chemical, physiochemical, or structural changes of the drug, protein, and/or both. The decrease in the degree of ionization in PF and 5-OH-PF (as demonstrated by a reduction in the fraction of PF and 5-
OH-PF in the aqueous layer of an organic solvent/buffer partition system) with increases in pH (Figure 5 and 6) would suggest that the binding of PF and 5-OH-PF is dependent to a large degree on hydrophobic rather than ionic interactions between the drug and the protein. It appears that since similar amounts of PF and 5-OH-PF are un-ionized at pH 7.4, other factors including, steric and chemical interactions due to the hydroxylation of PF, may account for the observed binding differences between the drug and metabolite.

The contribution of pH dependent changes in protein (conformational, chemical and physiochemical) to the drug-protein binding of PF and 5-OH-PF is not known. However, HSA has been shown to undergo pH dependent conformational transitions, which could potentially alter drug-protein binding [Kremer et al., 1988]. It is not known whether AAG undergoes pH sensitive changes affecting drug-protein binding [ionization, or conformational changes]. Thus, it seems likely that a pH increase induced decrease in the degree of ionization of both PF and 5-OH-PF contributes to their increased binding in serum and such isolated serum proteins as AAG and HSA. It is not clear to what degree, if any, pH mediated alterations of serum proteins (HSA, AAG, and lipoproteins) play in the observed pH dependent drug-protein binding of PF and 5-OH-PF.
4.1.5 THE EFFECT OF BUFFER STRENGTH ON THE DRUG PROTEIN BINDING OF 5-HYDROXYPROPAFENONE.

When pH dependent drug-protein binding is present, it is important to use a buffer with sufficient buffer capacity to control the pH during the course of dialysis [Lindup, 1987]. Since both PF and 5-OH-PF undergo significant pH dependent binding, an increase in buffer strength from 0.067 M to 0.100 M was required to maintain constant pH during the dialysis experiment. However, buffer strength and composition can have large effects on drug-protein binding as measured by equilibrium dialysis [Lindup, 1987; Kwong, 1985]. The addition of high concentrations of inorganic phosphate ions, 7 to 10 fold higher than in normal serum [Hansten, 1986], may result in conformational shifts in serum proteins which may alter the drug-protein binding. The introduction or removal of various inorganic ions in serum, as a result of equilibrium dialysis, may result in conformational changes in the protein which can alter drug-protein binding. It has been shown that both chloride ions [Kremer et al., 1985], and calcium ions [Lindup, 1987] alter drug-protein binding. As molar strength of the phosphate buffer is increased, the amount of sodium chloride required to maintain isotonicity is reduced by over 50%, leading to an overall reduction of the amount of chloride ion in the buffer. It has also been suggested that phosphate present in the buffer may bind calcium ions, and thus reduce the influence of calcium on drug-protein binding [Kwong, 1985]. The strength of phosphate buffer did not affect the binding of 5-OH-PF in serum (Figure 7); however, the same may not be true with respect to the drug-protein binding to isolated serum proteins, such as HSA and
AAG. Isolated proteins in solution do not have other proteins normally present in serum to accommodate the liberated free drug due to binding alterations; therefore, altered binding to individual serum proteins due to the presence of buffer may go unnoticed in serum.

4.1.6 DEGRADATION OF 5-HYDROXYPROPAFENONE DURING EQUILIBRIUM DIALYSIS

Impurities may result in highly erroneous drug-protein binding results due to the displacement of the drug of interest from its protein binding site by an impurity present in the dialysis system [Kristensen and Gram, 1982]. These impurities may be the result of drug degradation during dialysis. Although pure 5-OH-PF was used to conduct binding experiments, the possibility of impurities due to degradation of 5-OH-PF during dialysis required consideration. No degradation of 5-OH-PF was observed during the course of the drug-protein binding experiment.

4.1.7 VOLUME SHIFTS DURING EQUILIBRIUM DIALYSIS OF PROPAFENONE AND 5-HYDROXYPROPAFENONE.

Volume shifts, the movement of fluid from the buffer compartment to the serum compartment during equilibrium dialysis and the subsequent effects on the interpretation of drug-protein binding data has been reviewed at length [Lima et al., 1981]. Volume shifts are time dependent and occur as a result of osmotic differences between the buffer and serum compartments in an equilibrium dialysis cell. Large volume shifts can dilute proteins in the serum side of the dialysis cell, and thus lead to an underestimation of the degree of drug-protein
binding (e.g. a 30% underestimation in the binding of prednisolone has been observed) [Tozer et al. 1981]. The volume shifts measured for PF and 5-OH-PF, respectively, were approximately 5%, and would not be expected to result in an appreciable underestimation of binding. In a similar study with the amine antiarrhythmic drug, disopyramide, a volume shift of 12% was required to be able to detect a change in the drug-protein binding [Lima et al., 1983]. A lack of a significant volume shift in the present experiment can be further demonstrated by the minimal change in the protein concentration before and after dialysis (See Table 2).

4.1.8 OSMOLARITY MEASUREMENTS OF 5-HYDROXYPROPafenONE DRUG SOLUTIONS.

Since osmotic differences contribute significantly to volume shifts between buffer and serum compartments of dialysis cells, the effect of adding different amounts of drug (0.1 to 45.0 µg/mL 5-OH-PF) to the buffer compartment was considered. The fact that osmolarity remained relatively constant with the addition of various amounts of drug could help to explain the constant nature of the volume shifts regardless of the drug concentration studied.

4.1.9 PROTEIN LOSS DURING EQUILIBRIUM DIALYSIS OF PROPafenONE AND 5-HYDROXYPROPafenONE

The reduction in the concentration of protein may occur as a result of volume shifts [Lima et al., 1983; Tozer et al. 1981], non-specific binding of proteins to dialysis cell surfaces and the dialysis
membrane, and enzymatic or chemical degradation of the protein [Kwong, 1985]. The reduction in the protein concentration following dialysis in this experiment was minimal, with the exception of AAG dissolved in buffer (Table 2). Since an equimolar reduction in the concentration of AAG did not occur in serum, it may be speculated that either degradation of AAG during dialysis in the buffer, or non-specific binding of AAG to the dialysis cell, and/or membrane, rather than sample dilution due to volume shifts was responsible for the observed reduction in AAG following dialysis.

4.1.10 MEMBRANE INTEGRITY DURING EQUILIBRIUM DIALYSIS

Another possible complication of equilibrium dialysis may be protein leakage during dialysis as a result of membrane rupture and improper mounting of the dialysis membranes. The membrane used in these experiments proved to be robust, since very few post-dialysis buffer samples tested positive for protein. It should be remembered, that this test is visual rather than spectrophotometric, and therefore lacks sensitivity. Only large amounts of protein in the buffer can be detected; however, if a membrane ruptures, or leaks, the amount of protein in the buffer would be substantial (approximately half of the serum protein concentration), and therefore, the visual test would be adequate.

4.2 PROTEIN BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE
4.2.1 PROTEIN BINDING OF 5-HYDROXYPROPafenONE IN SERUM OF HEALTHY MALES

The drug-protein binding interaction can be described by the equilibrium in equation 1:

\[
[D_f] + [P] \rightleftharpoons [D*P]
\]

\[
\frac{k_1}{k_2}
\]

where \([D_f], [P], \) and \([D*P]\) are the concentrations of free drug, free protein, and bound drug and protein, respectively. This interaction between drug and serum protein can be described by the affinity association constant \((K_a)\) or the strength of the interaction as in equation 2.

\[
K_a = \frac{[D*P]}{[D][P]} = \frac{k_1}{k_2}
\]

Binding can also be described by the number of binding sites available, or capacity \((n[Pt])\) for drug in the drug-protein binding system where \(n\) is the number of binding sites on the protein, and \([Pt]\) is the total amount of protein present in the system. Upon manipulation, equation 2 can describe the binding of drug to protein by a binding isotherm (Equation 4).

\[
r = \frac{[D*P]}{[Pt]} = \frac{nK_a[D]}{1 + K_a[D]} \tag{4}
\]

where \(r\) is the molar ratio of drug bound. Upon multiplying by the total protein concentration \([Pt]\), equation 4 can be transformed to
represent the amount of bound drug (equation 5).

\[
[D*P] = \frac{n*\text{K}_a*[P_t]*[D]}{1 + \text{K}_a*[D]}
\] (for one binding site) \hspace{1cm} (5)

For multiple binding sites the contribution of each binding site is summed.

\[
[D*P]_{\text{total}} = \sum_{i=1}^{p} [D*P]_p
\] (for p number of binding sites) \hspace{1cm} (6)

The difficulty encountered in the estimation of binding parameters, due to the non-linear nature of equation 5 has resulted in the presentation of several transformations to linearize binding data, of which the most common are Scatchard [Scatchard, 1949], and Rosenthal [Rosenthal, 1967].

Scatchard transformation

\[
\frac{r}{D} = n*\text{K}_a - r*\text{K}_a
\] \hspace{1cm} (8)

Rosenthal transformation

\[
[D*P] = n*\text{K}_a*[P_t] - [D*P]*\text{K}_a
\] \hspace{1cm} (9)

Several problems can be encountered with these transformations. If more than two different homogeneous types of binding sites are present (Equation 6), the linear transformations becomes curved. Therefore, any advantage gained by transforming the data is lost, since the parameters cannot be determined graphically. The Scatchard and Rosenthal analysis also tend to be statistically invalid since the y-observations are not independent of the x observations [Burgisser, 1984]. Rosenthal and Scatchard plots, commonly used in describing binding data, may only provide a qualitative description of the binding interaction.
The alternative approach is nonlinear regression with computer programs, such as ENZFITTER\textsuperscript{R}, NONLIN\textsuperscript{R}, and LIGAND\textsuperscript{R}, of the untransformed data (Equation 4 and 5) [Burgisser, 1984]. This method does not rely on transformations which may emphasize data points at lower drug concentrations where the potential error is greater, such as the Klotz reciprocal plot [Svensson et al., 1986]. Further this method of fitting untransformed binding data is statistically rigorous, since all the y-observations are independent of x observations (eg. \( y = f(x) \) rather than \( y = f(y/x) \)) [Klotz, 1982]. Despite the advantages of fitting data by this method, it should also be considered that the accuracy of fitted parameters depends on the quality of the data [Klotz, 1983; Burgisser, 1984]. Thus, data with considerable scatter will not provide the most accurate estimates of the drug-protein binding parameters.

The binding affinities of the high capacity site on proteins in serum for PF and 5-OH-PF differ considerably (\( K_{a1}(PF) = 6.53 \times 10^5 \text{ M}^{-1} \) [Chan et al., 1989b] vs. \( K_{a1}(5\text{-OH-PF}) = 1.72 \times 10^4 \text{ M}^{-1} \)); however a similar binding capacity has been reported (\( N_{p1}(PF) = 1.73 \times 10^{-4} \text{ M} \) [Chan et al., 1989b] vs. \( N_{p}(5\text{-OH-PF}) = 3.46 \times 10^{-4} \text{ M} \)) (Table 3). This raises the question: Are the binding constants estimated for 5-OH-PF a mixture of two homogeneous binding sites, such that the observed Ka and NP are hybrid constants, or does 5-OH-PF in fact possess only one homogeneous type of binding site in human serum which has a lower affinity but similar capacity to that observed for PF in serum.

The drug-protein binding parameters determined by non-linear
regression of binding data in serum, such as binding affinity and capacity of binding sites, must be considered cautiously since these are macro constants composed of the sum of binding affinities and binding capacities of numerous proteins present in serum. Similarly, as numerous binding sites of similar affinities and capacities appear homogeneous in serum, if the data points are scattered, the binding affinities and capacities of two different homogeneous groups of binding sites may appear as one homogeneous site. This is particularly evident when the difference between the affinity for the high and low affinity binding sites is small. In figure 27 can be seen a theoretical demonstration of the effect of decreasing the binding affinity of the high affinity binding site (K_a), thus reducing the difference between the affinities for the high and low affinity binding site. The binding capacity of the high affinity binding site is unaltered, and the second low affinity site provides a constant contribution to binding. As the theoretical binding affinity is decreased, it becomes increasingly difficult to graphically distinguish between the two binding sites. Furthermore, if data is excessively scattered it may become difficult, if not impossible, for nonlinear regression to reliably differentiate between these two different binding sites. Such may be the case with 5-OH-PF binding. Analysis using the Rosenthal plot, in addition to nonlinear fitting of the untransformed data show only one homogeneous set of binding sites; however, there is considerable scatter in the data at lower concentrations (Figures 8a-8e). Therefore, binding constants reported for 5-OH-PF may not be completely accurate.

Another problem encountered during characterization of the binding
5-OH-PF was the poor solubility of this compound. While ethanolic solubilization of the drug might offer some advantages in certain experimental circumstances, the addition of 5% ethanol was found to affect the binding of perazine by 12% in a solution of AAG [Kremer et al., 1988]. To avoid similar interferences in the present binding study no exogenous chemicals were added. The maximum concentration of 5-OH-PF used in binding experiments was 45.0 µg/mL. This provides a smaller concentration range of 5-OH-PF, compared to PF, over which the binding can be characterized. This further increases the difficulty to experimentally differentiate between two different binding sites in serum [Klotz, 1982].

A gradual rise in the free fraction of 5-OH-PF was observed over the range of concentrations studied (Table 4); however, the gradual increase in free fraction could not be differentiated statistically. As the difference between the high and low affinity site affinity binding constants becomes smaller a rise in free fraction is less pronounced, whereas, if this difference is large, the free fraction would remain low until saturation of the high affinity binding site is reached (Figure 28). Following saturation of the high affinity binding site, the free fraction would show an abrupt increase. Such behaviour would suggest that the compound undergoes abrupt non-linear binding over a narrow range of concentrations, whereas, a gradual increase, as observed for
Figure 27. Theoretical Rosenthal plot of drug-protein binding fitting a binding model for a system containing one drug binding specific site + non-specific binding site. The binding capacity of the high affinity site is 1.0 and the non-specific binding (Kns) was held at 2; only the affinity of the high affinity site is altered.

Affinity constants Ka: 10 (○), 50 (△), and 200 (●).
Figure 28. Theoretical plot of free fraction of drug-protein binding fitting a model containing one specific site + non-specific binding. Binding constants (i.e. the capacity of the high affinity site is 1.0, and non-specific binding is 2) are held constant; only the affinity of the high affinity site is altered.

Affinity constants $K_a$: 10 (○), 50 (△), and 200 (●).
compounds were the difference in binding affinities of the low and high affinity sites are small, may not appear non-linear even though the high affinity site is also saturated. Although the free fraction of 5-OH-PF at higher concentrations does vary statistically, the free fraction at the lowest concentration (0.1 µg/mL) was shown to be statistically different compared to the other observed values. The exact reason for this is not known; however, considering that drug concentration measurements, particularly from the buffer compartment, were close to the limit of sensitivity of the assay method, increased experimental error in measuring the 5-OH-PF concentration may have contributed to this low observation.

4.2.2 PROTEIN BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE IN SERUM ESTIMATED BY EQUILIBRIUM DIALYSIS AND ULTRAFILTRATION.

The drug-protein binding observed when control serum is spiked with a drug, may not correlate well with the drug-protein binding of the same drug when binding is measured following the systemic administration of the drug. One possible explanation for this may be that the binding of the parent compound is inhibited by the metabolite following systemic administration of the drug. For example, the binding of both disopyramide and verapamil were found to decrease by the addition to their respective metabolites, mono-n-dealkylated disopyramide [Bredensen et al., 1982], and norverapamil [Bates et al., 1980]. The possibility of decreased binding of PF due to the presence of metabolite (5-OH-PF), in addition to a slightly higher concentration of AAG may help explain the difference in free fraction at therapeutic
concentrations of PF observed earlier [Chan et al. 1989b] (0.041 ± 0.011), and results currently reported (0.063 ± 0.004). In the present experiment therapeutic concentrations of propafenone (2.0 μg/mL) and 5-hydroxypropafenone (0.5 μg/mL) which are normally seen following multiple oral dosing were used [Kates et al., 1985]. Conversely, if metabolite binding is investigated, the parent compound may displace the metabolite from drug-protein binding sites in serum. The drug-protein binding of 5-OH-PF appeared unaltered by the addition of therapeutic concentrations of the parent drug, PF. This was unexpected, considering the differences in binding affinity between PF and 5-OH-PF to serum proteins, however, other serum proteins may be able to accommodate the liberated 5-OH-PF.

Ultrafiltration consistently overestimated the binding of both PF and 5-OH-PF. One explanation for the consistent overestimation of binding by ultrafiltration could be the high non-specific binding of both PF and 5-OH-PF which was not corrected for, in the ultrafiltration study. However, even if the non-specific binding contribution is estimated and included, the binding of PF is still overestimated compared to equilibrium dialysis.

The temperature difference between ultrafiltration and equilibrium dialysis could offer another possible explanation for the overestimated binding observed with ultrafiltration as compared to equilibrium dialysis. The drug-protein binding interaction is mediated by hydrophobic interactions, ionic bonds, Van der Waal's forces, and hydrogen bonding [Laider et al., 1983; Laznicek et al., 1987; Lindup,
These binding interactions are all influenced, to a differing degree, by changes in temperature [Laider et al., 1983]. In fact, the association constant for reversible protein-ligand binding interactions can be written as a function of temperature, such that, as temperature is increased, the association constant decreases [Laider et al., 1983]. This would suggest that as the temperature of a drug-protein binding system is increased the drug-protein binding is decreased. This has been shown for drugs, such as propranolol [Paxton et al., 1983], quinidine [Nilsen and Odd, 1976], and warfarin [Oester et al., 1973]. Since ultrafiltration was carried out at 25 °C and equilibrium dialysis was carried out at 37 °C, temperature dependent binding could contribute to the observed difference in binding between ultrafiltration, and equilibrium dialysis. Temperature dependent binding, in addition to non-specific binding, could provide a reasonable explanation for the large observed difference in drug-protein binding between ultrafiltration and equilibrium dialysis.

4.3 BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE TO ISOLATED SERUM PROTEINS.

4.3.1 BINDING DIFFERENCES BETWEEN PROPafenONE AND 5-HYDROXYPROPafenONE IN VARIOUS PROTEIN SOLUTIONS.

Large differences in the free fraction are apparent between PF and 5-OH-PF in serum, lipoprotein deficient serum, and AAG solution (Figure 9). AAG appears to account for a large portion of the binding of PF at low concentrations, in the presence of the metabolite, 5-OH-PF. This is
not an unexpected observation since the binding of PF in serum was shown to correlate well with the amount of AAG present [Chan et al., 1989b]. In addition, it has been reported that PF binds with high affinity to AAG [Gilles et al. 1985]. Further, it was shown that AAG accounts for the majority of binding of the weakly basic amine drugs propranolol and prazosin at lower concentrations [Sager et al., 1989].

The binding of 5-OH-PF does not approach the extent of binding observed in serum for PF. This may be a result of chemical and/or conformational differences introduced to the drug molecule as a result of metabolism (i.e. the addition of a phenolic hydroxy group). This metabolism induced difference may increase the binding affinity of the metabolites as is the case with disopyramide [Bredensen et al., 1982], or conversely metabolism may reduce the binding affinity of the metabolite as suggested for 5-OH-PF (Table 4). The extent of 5-OH-PF binding to AAG may also be reduced due to displacement by the parent compound, PF. In fact, the binding of 5-OH-PF to AAG in the presence of PF is lower than that observed when PF is absent (FF without PF 0.32 ± 0.08, and FF with PF is 0.55 ± 0.10). Thus, chemical and steric differences may reduce the binding affinity of 5-OH-PF, and subsequently make it more prone to displacement from AAG by the parent drug PF.

The binding of PF and 5-OH-PF in a solution of isolated HSA is low, despite the fact that an approximate 30 fold greater molar concentration of HSA compared to AAG was used, thereby providing a much greater abundance of potential drug-protein binding sites. The majority of PF, at the low concentrations studied, was bound to AAG, whereas the
5-OH-PF shows much lower binding to AAG. This would suggest that HSA is relatively more important in the binding of 5-OH-PF as compared to PF at therapeutic concentrations (−2.0 μg/mL of PF and −0.5 μg/mL 5-OH-PF).

It would appear that a solution of isolated AAG at a similar concentration to that encountered in normal human serum can account for the same degree of drug-protein binding of PF found in serum at the low concentrations studied (Figure 9). Thus, the addition of physiological concentrations of HSA would not be expected to alter the binding; however, when such an experiment was conducted an increase in the free fraction was observed (Figure 9). The reason for this is not known; however, one explanation could be that the addition of impurities introduced by the HSA due to purification may play a role. Heavy metal ions such as mercury, silver, copper, and iron have been shown to decrease the binding of various ligands to AAG in a non-competitive manner [Kerkay and Westphal, 1969]. Alternatively, protein-protein interactions of these isolated proteins in a buffer solution may result in altered conformation and therefore account for the observed decrease in binding. Although the pH in the pure AAG solution is higher than that in the AAG + HSA solution (ie. as pH increases, binding increases), this difference in pH could not account for the observed difference in binding.

The addition of physiological concentrations of HSA to AAG decreased the free fraction of 5-OH-PF. This provides further evidence that HSA, in addition to AAG, is an important binding protein for 5-OH-PF at the concentrations studied. The free fraction of 5-OH-PF observed
in the AAG + HSA solution approximated the free fraction observed in the lipoprotein deficient serum (Figure 9).

It would appear that the binding of PF at therapeutic concentrations could be accounted for largely by AAG, similar to the binding profiles observed for propranolol [Glasson et al., 1980; Sager et al., 1989]. In contrast, binding of 5-OH-PF appears to be more dependent on various other serum proteins as seen with quinidine [Nilsen et al., 1976]. The reason for the difference in the degree to which PF and 5-OH-PF bind to varying degrees to different serum proteins is not clear. However, chemical, physiochemical, and structural differences due to metabolism, and subsequent displacement of 5-OH-PF by PF, due to its lower affinity, from AAG may result in the binding of 5-OH-PF becoming more dependent on other serum proteins at low concentrations.

4.3.2 THE EFFECT OF BUFFER AND SERUM ULTRAFILTRATE ON THE BINDING OF PROPAFENONE AND 5-HYDROXYPROPafenONE.

Buffer composition and strength can have significant effects on the binding of various ligands, as discussed previously (Section 4.1.6) [Kremer et al., 1988; Lindup, 1987]. The difference in binding of PF between buffer and serum ultrafiltrate in the AAG + HSA solution may be, in part, due to differences in pH between the two solutions (Figure 10). That is, with an increase in pH, the binding of PF in the AAG + HSA dissolved in ultrafiltrate solution increases to yield a lower observed free fraction. However, pH sensitive binding is not a factor in the observed differences for the drug protein binding of PF between AAG
dissolved in either the buffer or serum ultrafiltrate. Other factors such as the differences in ionic composition, and strength likely play a role. The lack of a statistically significant difference in the free fraction of 5-OH-PF between AAG dissolved in either buffer or serum ultrafiltrate could be a result of the variability in the binding measurements. This lack of a difference for 5-OH-PF binding between AAG dissolved in either serum ultrafiltrate or the buffer could also be a result of the relatively minor importance of AAG in the binding of 5-OH-PF compared to PF. It appears that the composition of the solvent used to dissolve AAG affects the drug-protein binding of PF but not 5-OH-PF. This would tend to suggest that AAG is more sensitive to non-physiological solvents, such as 0.1 M phosphate buffer, since the binding of PF (highly bound to AAG) was affected by buffer composition, whereas the binding of 5-OH-PF (slightly bound to AAG) was unaltered.

4.3.3 THE EFFECT OF THE REMOVAL OF FREE FATTY ACIDS FROM ALBUMIN ON THE BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE.

Free fatty acids of various chain lengths bind to various sites on the HSA molecule [Krahg-Hansen, 1981]. Increases in the concentration of free fatty acids have been shown to decrease the binding of a number of different endogenous ligands such as tryptophan [Krahg-Hansen, 1981], and various drugs such as, diazepam [Vallner, 1977] and warfarin [Nilsen et al., 1974]. Conversely, free fatty acids may also have a stabilizing effect on the HSA molecule and result in increased binding of certain ligands such as testosterone and progesterone [Krahg-Hansen, 1981]. The binding difference of propafenone between HSA and free fatty acid free
HSA may be partially explained by differences in pH between the two groups (Figure 12). That is, as pH increases, binding increases in a proportionate manner over the pH range studied (Figure 3 and 4). Since the pH in the free fatty acid group was greater, a decrease in free fraction was expected. This observed difference in the binding between free fatty acid free HSA and HSA may be an artifact of pH, and there may in fact be no difference in the binding between free fatty acid free HSA, and HSA. Similarly, the addition of the free fatty acid, palmitic acid, did not affect the binding of propranolol to HSA until a 10:1 ratio of free fatty acid to HSA was achieved [Glasson et al., 1980]. In contrast to PF, it is unlikely that pH is the only contributing factor involved in the observed binding differences of 5-OH-PF, since similar pH dependent differences in binding were not observed for the binding of 5-OH-PF.

4.3.4 THE EFFECT OF THE REMOVAL OF LIPOPROTEINS FROM SERUM ON THE BINDING OF PROPafenONE AND 5-HYDROXYPROPafenONE.

Lipoproteins play a role in the binding of many basic and neutral drugs including various tricyclic antidepressant agents [Routledge, 1986], propranolol [Tillement et al., 1980], tianeptine [Tillement et al., 1990] and quinidine [Nilsen et al., 1976]. Since PF binding in serum at the concentrations studied appears to be dominated by AAG, the removal of lipoproteins was not expected to result in a difference in binding (Figure 13). Unlike PF, it appears that drug-protein binding of 5-OH-PF is dependent on a number of different serum proteins; therefore, the removal of lipoproteins was expected to result in an increase in the
free fraction of 5-OH-PF. The removal of lipoproteins leads to an approximately 40% increase in the metabolite free fraction at the concentrations studied. This suggests that the increase in free 5-OH-PF could not be accommodated by additional binding by HSA and AAG, in the presence to PF. This observation provides further evidence for differences between the binding profiles of the parent drug and metabolite when present together at therapeutic concentrations. That is, the binding of PF 2.0 μg/mL in the presence of the metabolite, 5-OH-PF, 0.5 μg/mL is dominated by AAG, whereas the binding of 5-OH-PF in the presence of PF is dependent on all of the investigated serum proteins (AAG, HSA, and lipoproteins).

4.3.5 DISPLACEMENT OF PROPAFENONE AND 5-HYDROXYPROPafenONE IN SERUM BY DISPLACING AGENTS IBUPROFEN AND DISOPYRAMIDE.

Ibuprofen has a high affinity for HSA [Kragh-Hansen, 1981]; therefore, it is not surprising that the addition of ibuprofen in serum at a therapeutic concentration (~40 fold greater than the molar concentration of PF) increased the free fraction of both PF and 5-OH-PF in serum (Table 6). These increases in free fraction may actually be misleading, since they do not correspond to the actual amount of drug displaced (ie. a 35% increase in free fraction of PF corresponds to a ~3% decrease in the bound fraction of PF whereas a 27% increase in the free fraction of 5-OH-PF corresponds to a ~10% decrease in the bound fraction). The larger decrease in the bound fraction of 5-OH-PF compared to PF may be due to the fact that HSA, as shown previously, may be more important in terms of binding 5-OH-PF at therapeutic
concentrations.

Disopyramide has been shown to bind to AAG with a large affinity [Kremer et al., 1988]. The addition of disopyramide as a displacing agent specific for AAG, was expected to increase the free fraction of PF to a larger extent, since it appears that AAG is important to the binding of PF in serum. The decrease in binding for both PF and 5-OH-PF was approximately -7%. This demonstrates that both PF and 5-OH-PF may appear to be displaced from AAG to a similar degree. Since 5-OH-PF may already have been displaced from AAG by PF, thus the addition of disopyramide is not expected to result in any further large increase in the free fraction of 5-OH-PF. Further, the increases in free PF and 5-OH-PF liberated from the binding sites may be accommodated by other low affinity proteins in serum such as HSA and lipoproteins (low affinity, high capacity) maintaining the free fraction of PF and 5-OH-PF observed in serum relatively constant.

4.3.6 DISPLACEMENT OF PROPafenONE AND 5-HYDROXYPROPafenONE FROM INDIVIDUAL HUMAN SERUM PROTEINS.

As anticipated, a large percentage of bound of PF was displaced from AAG (increase in free fraction) with the addition of disopyramide; however, a similar decrease in binding (increase in free fraction) was not observed for 5-OH-PF. Because PF may already have displaced significant quantities of 5-OH-PF from AAG binding sites, the further addition of disopyramide may not result in a further increase in the
free fraction of 5-OH-PF. Furthermore, the larger variability of the binding measurement for 5-OH-PF may also overshadow a smaller difference in the binding of 5-OH-PF to AAG due to the addition of disopyramide.

Ibuprofen, used as a displacing agent for HSA, also increased the free fraction of PF bound to AAG. This can be explained by the large difference in the concentration of PF and ibuprofen, and that acidic drugs are also known to bind to AAG to a certain degree [Urien et al., 1982]. Most acidic drugs bind to AAG with a much lower affinity than that commonly reported for basic and neutral drugs. It was found that drugs that bind to site I of HSA and also lack a carboxylic group generally bind to AAG with a higher affinity than acidic drugs containing a carboxylic group [Urien et al., 1982]. Since ibuprofen contains such a carboxylic group it is speculated that it should bind to AAG with a low affinity; however, this has never been demonstrated. The extent of binding is dependent on both the concentration of ibuprofen and the affinity; therefore, despite the lower affinity of ibuprofen for AAG, ibuprofen may still bind. Thus, the increase in free fraction of PF observed in the isolated solution of AAG may be due to ibuprofen competitive, or non-competitive binding displacement of PF. Although 5-OH-PF also shows a decrease in binding with the addition of ibuprofen, this difference does not reach statistical significance.

The binding of both PF and 5-OH-PF to HSA was shown to increase with the addition of the amine drug, disopyramide (decreasing free fraction). The reason for this observation was not explained. Since it is unlikely that conformation changes in HSA will occur due to the
binding of disopyramide to HSA, this suggests that differences in pH between the control and disopyramide treatment groups may partially contribute. A similar, but unexpected, observation was made for 5-OH-PF binding to HSA with the addition of ibuprofen. Ibuprofen may bind to a different site of the HSA molecule than 5-OH-PF, and therefore cause conformational shifts which could possibly expose hydrophobic sites which can further bind 5-OH-PF present in buffer. This appears to be true only in buffer and not in serum, since the free fraction in serum was increased when ibuprofen was added. Another explanation, although unlikely, is that the displacement of 5-OH-PF may occur from other proteins rather than HSA and AAG, as originally speculated. The free fraction of PF in a solution of HSA was found to increase with the addition of ibuprofen, as originally expected (Figure 14). The results for PF in isolated proteins HSA and AAG are qualitatively similar to the observations in serum.

4.4 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE TO ISOLATED PROTEINS

4.4.1 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE IN A SOLUTION OF AAG.

Binding of PF to AAG appears to be quite specific and saturable, similar to that shown for propranolol, PF, lidocaine, verapamil [Gilles et al., 1985], propranolol [Glasson et al., 1980], isoproterenol, norepinephrine, epinephrine [Sagar et al., 1987], and many other basic drugs [Muller et al., 1983]. Much controversy exists as to the number
of binding sites for drugs on AAG. Many authors report only one binding site from indirect binding measurements, whereas others report two different drug-protein binding sites using direct binding studies (e.g. one high affinity, low capacity binding site, and one low affinity high capacity site.) [Kremer et al., 1988]. In agreement with earlier results [Gilles et al., 1985], the binding of PF to AAG appears to have two different binding sites (Figure 16a). Although it is generally not valid to compare binding affinities from different studies, qualitative observations between experiments conducted in similar experimental conditions may be appropriate. The magnitude of the high affinity binding site for PF reported earlier, correlated well with the affinity constant measured [Gilles et al., 1985]. The 6 fold difference measured between the affinity of the present study and the previous reported value could likely be due to differences in experimental technique [Kremer et al., 1988] (i.e. a rigorous method for the further purification of AAG was conducted in the earlier study [Gilles et al., 1985]). Thus, direct comparisons between binding parameters must be made with caution. In contrast to the earlier studies [Gilles et al., 1985], a 10 fold larger difference in the capacity and a 35 fold decrease in the affinity of the low affinity binding site for PF on AAG were measured [Gilles et al., 1985]. Binding of isoproterenol, norepinephrine, and epinephrine show a low affinity site with a large capacity, similar to what has been observed for the low capacity site for PF [Sager et al., 1987]. However, despite the findings for catecholamines, the capacity for PF at the low affinity site is suspiciously high.
The study of 5-OH-PF binding to AAG did not suggest the presence of more than one drug-protein binding site. This may result from the relatively small difference between the high and low affinity binding site on AAG. If the affinities for these sites closely approximate each other, it may be difficult if not impossible to distinguish between the two binding sites. This would result in hybrid binding constants, as discussed earlier (Figure 16b). This, however, may not be the case for 5-OH-PF since the capacity of the high affinity site of PF is similar to that observed for 5-OH-PF. The major difference between these two sites is the binding affinity (Kal PF is 10 fold greater than the observed Ka for 5-OH-PF). Since the addition of 2.0 µg/mL of PF to a solution of 0.5 µg/mL 5-OH-PF decreases the metabolite binding by ~20%, it seems likely that PF and 5-OH-PF share a common binding site on AAG. This displacement appears to be competitive in nature since the addition of 0.5 µg/mL of 5-OH-PF to 2.0 µg/mL of PF results in a displacement of ~2% (ie. the displacement is mutual, but PF with the higher affinity constant appears to displace 5-OH-PF more readily. At first this would not appear to correlate well with the previously observed results suggesting that 5-OH-PF is not highly displaced by disopyramide from AAG. However, it should be considered that PF present in the earlier studies may already have displaced a significant quantity of 5-OH-PF prior to the addition of disopyramide.

If changes in free fraction are considered, as the concentration of drug increases and approaches the capacity of AAG, the free fraction, as expected from figure 28, demonstrates an abrupt rise. This abrupt increase in the free fraction of both PF and 5-OH-PF occurs as the
concentration surpasses the capacity of the high affinity site on AAG (Figure 22 and 23).

4.4.2 THE BINDING CHARACTERISTICS OF PROPAFENONE AND 5-HYDROXYPURPRAFENONE IN A SOLUTION OF HUMAN SERUM ALBUMIN.

As with many basic drugs, such as, propranolol [Glasson et al., 1980], quinidine [Nilsen, 1976], and various catecholamines [Sager et al., 1987], both PF and 5-OH-PF display non-saturable binding to HSA (Figure 16a and 16b). The binding affinity measured for PF and 5-OH-PF to AAG is 6000 and 1500 fold greater that the affinity to HSA for PF and 5-OH-PF, respectively. The low affinity and high capacity binding sites are in keeping with HSA binding observed for various other basic or neutral compounds [Kremer et al., 1988]. Since a high capacity site was observed for PF and 5-OH-PF to HSA, concentration dependence in the binding of 5-OH-PF and PF would not be expected as was observed (Figures 22 and 23).

4.4.3 CHARACTERIZATION OF PROPAFENONE AND 5-HYDROXYPURPRAFENONE BINDING IN SERUM.

PF shows two binding heterogeneous sites in serum (Figure 18a). This would be expected considering the ~6000 fold difference in affinities between AAG and HSA. The high affinity binding site in serum is approximately 30 fold lower than that in pure AAG. This could be a result of endogenous competitive or non-competitive binding inhibitors present in serum. Another explanation could be that since the high
The same relationship observed for PF does not hold for 5-OH-PF since only one binding site was apparent for the metabolite in serum (Figure 18b). It has been shown for 5-OH-PF that a high affinity site present on AAG is approximately 1500 fold greater affinity than the low affinity site present on HSA; therefore, a similar relationship as seen for PF in serum was expected. However, competitive and non-competitive binding inhibitors present in serum could result in this difference being reduced substantially, as was seen with PF. This, in turn, could result in the difference between the high and low affinity binding constants being too small to detect two distinct binding sites (Figure 27). It is highly likely that 5-OH-PF, without the addition of PF, displays a similar binding profile to PF in serum. However, the inherent scatter in the 5-OH-PF data, in addition to the small difference between the high and low affinity binding sites results in the inability of one to experimentally differentiate the presence of two binding sites in serum. Thus, the reported binding constants for 5-OH-PF in serum are likely to be a hybrid constant of two different binding sites.

PF undergoes concentration binding; an abrupt rise in the free
fraction is observed as the concentration increases past the capacity of the high affinity binding site (Figure 22). Since the capacity of the high affinity site correlates well with the capacity of AAG, the concentration dependent binding of PF can be likely attributed to the saturation of AAG. Although a similar saturation of AAG by 5-OH-PF is evident, concentration dependent binding in serum is not apparent. Since the Ka for 5-OH-PF is significantly less than that of PF, the gradual rise in free fraction predicted in figure 28 may not be apparent when compared to the abrupt rise in free fraction of PF with increasing total drug concentration in serum. The inherent scatter of the 5-OH-PF free fraction data may result in an increase in free fraction being overshadowed. Since it was shown that other proteins in the serum bind 5-OH-PF, these other proteins (HSA and lipoproteins) may accommodate the 5-OH-PF once it has saturated AAG.

4.4.4 BINDING CHARACTERISTICS OF PROPafenONE AND 5-HYDROXYPROPafenONE TO LIPOPROTEINS.

Lipoproteins are complex mixtures of proteins and varying amounts of lipid. In addition, these complexes differ significantly between various classes of lipoproteins (see Table 7).
TABLE 7: THE COMPOSITION OF DIFFERENT CLASSES OF LIPOPROTEINS [VLDL, LDL, AND HDL]

<table>
<thead>
<tr>
<th>COMPOSITION</th>
<th>VLDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOTAL LIPID</td>
<td>90%</td>
<td>75%</td>
<td>50%</td>
</tr>
<tr>
<td>TRIGLYCERIDES</td>
<td>60%</td>
<td>10%</td>
<td>11%</td>
</tr>
<tr>
<td>CHOLESTEROL</td>
<td>17%</td>
<td>60%</td>
<td>32%</td>
</tr>
<tr>
<td>PHOSPHOLIPID</td>
<td>20%</td>
<td>30%</td>
<td>50%</td>
</tr>
<tr>
<td>TOTAL PROTEINS</td>
<td>-12%</td>
<td>-25%</td>
<td>-50%</td>
</tr>
</tbody>
</table>

APO-LIPOPROTEIN TYPE

<table>
<thead>
<tr>
<th>MAJOR</th>
<th>B100</th>
<th>B100</th>
<th>A-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>C’S</td>
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<td>-</td>
<td>A-II</td>
</tr>
<tr>
<td>E</td>
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<table>
<thead>
<tr>
<th>MINOR</th>
<th>A-I</th>
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<th>C’S</th>
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<tr>
<td>A-II</td>
<td>E</td>
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<td>D’S</td>
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<tr>
<td>B48</td>
<td>-</td>
<td>E</td>
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</tbody>
</table>

TABLE 7 FROM Scanu, (1985)

Considering the diversity of various classes of lipoproteins, it is not unreasonable to expect different degrees of drug-protein binding or in the case of lipoproteins, solubilization, in various lipoproteins depending on the characteristics of the drug in question. Many basic and neutral drugs, such as catecholamines [Sager et al., 1987], quinidine [Nilsen, 1976], amitriptyline, nortriptyline, doxepin, desmethyldoxepin [Pike et al., 1984], tianeptine [Zini et al., 1990], and propranolol [Glasson et al., 1980] bind to lipoproteins with differing degrees. It is generally found that drug binding to lipoproteins follows the general order of importance: HDL > LDL > VLDL [Glasson et al., 1980; Zini et al., 1990].
PF binding to HDL unlike previous studies with other basic drugs appears to have a saturable binding site within the HDL complex (Figure 19a). This saturable binding of PF to the HDL complex could be due to the large concentration of proteins in the HDL complex as compared to the other complexes. It should also be considered that the non-specific binding contribution to PF HDL-binding may have been obscured due to the limited solubility of PF in pH 7.4 buffer; therefore, binding could not be characterized in concentrations of PF beyond 100 μg/mL to completely saturate the apparent saturable binding site on the HDL complex.

The binding of both PF and 5-OH-PF to the LDL complex was extremely high. Results from figures 22 and 23 suggest the LDL could account for a larger degree of binding than observed for both PF and 5-OH-PF in serum. The reason for this is not known, but it may be speculated that the binding interaction of PF and 5-OH-PF with LDL is one of solubilization of PF and 5-OH-PF, rather than the characteristic drug-protein interaction. In fact, non-specific binding was shown for account for the binding interaction of PF and 5-OH-PF to both LDL and VLDL. Non-specific binding is the product of the capacity and the binding affinity constant [Glasson et al. 1980]. Since the capacity of LDL has been shown to be large, it is highly likely that the binding affinity constant is very low. Binding of PF and 5-OH-PF to VLDL was very low in comparison to LDL. The metabolite, 5-OH-PF, displayed almost a 10 fold greater binding to VLDL than the parent drug, PF. If the interaction of PF and 5-OH-PF with VLDL is one of solubilization of the drugs rather than classical drug-protein binding, the higher solubility of 5-OH-PF in hexane and toluene at pH 7.4 would suggest that
5-OH-PF can partition into the VLDL with greater ease than PF, due to the chemical differences between the parent drug and metabolite. It is likely that the interaction of PF and 5-OH-PF with lipoproteins is largely due to solubilization since the log of non-specific binding (Ka*NP) shows a very good correlation (r=0.92 for PF and r=0.98 for 5-OH-PF) to the amount of cholesterol present in the lipoprotein complex (i.e. LDL has the highest concentration of cholesterol in the complex followed by HDL and VLDL [see table 7]).

This demonstrates that lipoproteins play a role in the binding/solubilization of both PF and 5-OH-PF. The drug-protein interaction to the lipoproteins studied would likely be due to solubilization and in some cases, such as PF and HDL binding, the classical drug-protein interaction.

4.4.5 CALCULATED BINDING OF PROPAFENONE AND 5-HYDROXYPROPAFENONE BY MATHEMATICAL RECONSTITUTION OF THE DRUG-BINDING SYSTEM.

The comparison of data obtained in protein solutions dissolved in buffer cannot be quantitatively compared to that obtained in serum, since serum is a complicated solution/suspension of various proteins, endogenous chemicals, and ions. However, qualitative comparisons may be useful in determining the relative importance of various proteins at specific concentrations of PF and 5-OH-PF. The calculated binding of PF and 5-OH-PF are greatly overestimated (figures 24 and 25), this appears to be largely due to the very high apparent solubilization of drug in LDL. As the contributions of LDL and VLDL to the overall binding of PF-
and 5-OH-PF are removed, the binding more closely correlates to that observed in serum. The reason for the large uptake of drug by the LDL complex is not completely known; however, it is speculated that the binding to LDL appears to be a solubilization phenomena of very high capacity. Without other proteins available to bind drug, and thus counteract forces of the solubilization of drug into LDL, the LDL complex appears to act as a reservoir for drug. That is, the LDL complex takes up large quantities of both PF and 5-OH-PF. When other proteins with higher affinity are not present, drug enters the lipoprotein complex unimpeded until the concentration gradient, the driving force for solubilization, is reduced. Another explanation could be that the model chosen is a gross over simplification of the actual binding process. That is, binding contributions for individual proteins are calculated independent of one another when, in fact, binding is a dynamic equilibrium between free drug, and all the serum proteins are involved in binding of drug. Endogenous binding inhibitors and enhancers present in serum, but not in our binding model, may also have to be taken into consideration.

AAG, by itself, at lower concentrations appears to be important in the binding of both PF and 5-OH-PF; conversely, the binding of 5-OH-PF appears to correlate better with that observed in serum with the addition of HSA at intermediate concentrations. No difference is apparent in the binding of PF with the addition of HSA. This appears to suggest that HSA and AAG are important binding proteins in serum for 5-OH-PF at lower concentrations, whereas only AAG appears to be important in the binding of PF at lower concentrations.
At high concentrations of 5-OH-PF it is found that the HSA and AAG contributions to binding correlate well with the serum, but tend to underestimate the binding observed. This tends to suggest that lipoprotein binding may be important at high concentrations of 5-OH-PF. Curiously, it appears that HDL plays a role in the binding of PF at intermediate and high concentrations, but still underestimates the binding of PF. This would tend to suggest that lipoproteins play an important role in the solubilization of PF at high concentrations. At first these results may appear to contradict the earlier observations, stating that AAG had a minor role in the binding of 5-OH-PF, but it should be considered that PF, which can displace 5-OH-PF from AAG, was present in the early studies.

4.5 UPTAKE OF PROPAFENONE AND 5-HYDOXYPROPAFENONE BY HUMAN RED BLOOD CELLS.

The time required for distribution of PF and 5-OH-PF into human red blood cells (RBC) was very rapid, similar to that reported for disopyramide [Garrett et al., 1985], and propranolol [Ogata et al., 1984]. The extent of uptake into red blood cells suspended in only buffer was very high, with 5-OH-PF distribution statistically higher than that of PF. This observation parallels the observation that 5-OH-PF distributes into VLDLs more readily than PF, and further the partitioning of 5-OH-PF in hexane at pH 7.4 is much greater than PF. The addition of serum proteins decreases the driving force for drug to distribute into the red blood cells. This is similar to the situation
speculated for large binding of PF and 5-OH-PF to LDL in buffer. The drug concentration ratio in RBC/supernatant (serum) for PF and 5-OH-PF corresponds well with the drug concentration ratio in RBC/supernatant of observed in whole blood. The whole blood ratio for PF was similar to that obtained for disopyramide [Garrett et al., 1985] and propranolol [Ogata, 1989]. The ratio observed for 5-OH-PF was almost twice that for PF, this may be due to the observed chemical differences between these two compounds as a result of metabolism.

4.6 SUMMARY

Due to the high degree and variability of non-specific binding displayed by PF and 5-OH-PF, equilibrium dialysis was felt to be the superior method for the determination of drug-protein binding for these two compounds. Many factors must be optimized and controlled in order for equilibrium dialysis to provide both accurate and reproducible estimates of drug-protein binding. The time to equilibrium was optimized, and many possible factors which could introduce significant error to the binding estimate were considered.

Both PF and 5-OH-PF were found to display pH dependent binding in serum and in a solution of isolated AAG. Although PF showed pH dependent binding in a solution of isolated HSA, the same was not true for 5-OH-PF. The pH dependent binding of both PF and 5-OH-PF appeared to correlate well with the degree of ionization, and the partitioning between toluene and phosphate buffer, as a function of pH. This suggests that the binding of both PF and 5-OH-PF is largely hydrophobic
(i.e. as the degree of ionization decreases the binding increases).

The addition of the parent drug, PF, did not appear to alter the binding of 5-OH-PF.

AAG appeared to account for the majority of PF binding even in the presence of the metabolite, 5-OH-PF. However, it appears that the addition of PF decreases the binding of 5-OH-PF to AAG. There appears to be no difference in the binding of PF with the addition of 5-OH-PF in HSA, and vice versa. The removal of lipoproteins from serum does not reduce the binding of PF; however, large decreases in the binding of 5-OH-PF were observed.

The binding characteristics of PF and 5-OH-PF were determined in solutions of AAG, HSA, HDL, LDL, and VLDL. Both PF and 5-OH-PF showed a high affinity, low capacity binding site on AAG; however, PF was shown to exhibit a second low affinity, high capacity binding site. The affinity for PF to the high affinity, low capacity site on AAG was approximately 10 fold greater than that observed for 5-OH-PF. The binding of PF and 5-OH-PF to HSA showed one low affinity, high capacity binding site. The difference in affinity and capacity between this site for PF and 5-OH-PF was small. The binding of PF and 5-OH-PF to HDL, LDL, and VLDL appeared to be due to solubilization rather than a "true" drug-protein binding interaction, since the degree of uptake of either PF and 5-OH-PF was closely correlated to the amount of cholesterol present within the lipoprotein complex. The only exception was PF binding of HDL, which appeared to display saturable binding. When
similar curve fitting was carried out in serum, as expected, PF showed two binding sites, one high affinity, low capacity, and one low affinity, high capacity. Although a similar situation was expected for 5-OH-PF, since it also showed a high affinity binding site on AAG and a number of lower affinity binding sites in solutions of isolated proteins, only one site could be described. It is highly likely that due to inherent scatter of the data, in addition to the relatively small difference between the affinities for the low and high affinity site, two binding sites in serum could not be experimentally distinguished. Thus, the binding parameter estimated for 5-OH-PF in serum was a hybrid macro constant of two theoretical binding sites. The addition of disopyramide further demonstrates that PF and 5-OH-PF may share a common binding site on AAG, since the addition of disopyramide displaced PF from AAG, but displaced relatively little 5-OH-PF. However, it should be considered that the 5-OH-PF was most likely displaced by PF prior to the addition of disopyramide. The uptake of 5-OH-PF by red blood cells was two fold greater than the uptake of PF in whole blood. This correlates well with the partitioning of both PF and 5-OH-PF between hexane and buffer (5-OH-PF was found to partition better into the hexane at pH 7.4 than PF).

Thus, it appears that PF binding in serum at therapeutic concentrations is dictated by AAG, with HSA and lipoproteins available to buffer free PF liberated as a result of a potential displacement interaction, or saturation of the AAG high affinity site. It would further appear that alterations in the concentration of AAG can have marked effects on the free fraction of PF, and therefore, its
pharmacological effect. The addition of 5-OH-PF does not appear to alter the binding of PF to AAG significantly. The addition of a phenolic 5-hydroxyl group to propafenone as a result of metabolism decreases the affinity of 5-OH-PF for AAG, in addition to the four fold greater concentration of PF to 5-OH-PF, results in the displacement of 5-OH-PF by PF. As a result of this competition for AAG, the binding of 5-OH-PF is distributed more to other serum proteins, such as HSA, and lipoproteins. Thus, HSA and lipoproteins, present in serum account for a significant degree of the binding of 5-OH-PF at therapeutic concentrations when PF is present. The binding of 5-OH-PF to HSA and lipoproteins appears to be largely non-specific. Therefore, it is unlikely that displacers will further increase the free fraction of 5-OH-PF. Furthermore, the binding of 5-OH-PF would appear to remain relatively constant with fluctuations in the concentration of AAG when compared to PF.

Since the free fraction of 5-OH-PF is approximately three fold greater than PF, it appears that 5-OH-PF may play a significant role in overall pharmacological effect following administration of PF systemically. However, it should be considered that in addition to 5-OH-PF, n-depropyl PF the other active metabolite, may also accumulate and contribute to the pharmacological effect of PF [Kates et al., 1985].
5. CONCLUSION

1. A equilibrium dialysis method was established to measure the drug-protein binding of PF and 5-OH-PF in human serum, and in solutions of isolated human serum proteins (AAG, HSA, HDL, LDL, and VLDL). This method was found to be both reproducible and correlate well with previous binding studies using PF. The drug-protein binding of both PF and 5-OH-PF was found to be pH dependent. Other possible sources of error were minimized, or appeared to be relatively insignificant.

2. The free fraction of 5-OH-PF was found to be 0.23 ± 0.02 and did not change significantly with the addition of the parent drug, PF.

3. At therapeutic concentrations (2.0 μg/mL PF with 0.5 μg/mL 5-OH-PF), PF appears to bind mainly to AAG. The binding of 5-OH-PF to AAG was much lower than that observed for PF. The binding of PF and 5-OH-PF to albumin was low. The removal of lipoproteins from serum decreased the binding of 5-OH-PF, whereas no change was seen for PF.

4. Disopyramide and ibuprofen displaced both PF and 5-OH-PF in serum. The addition of disopyramide to a solution of isolated AAG and HSA resulted in an increase in the free fraction of PF, whereas the
free fraction of 5-OH-PF was unaltered. Ibuprofen was found to
decrease the free fraction of 5-OH-PF and PF in a solution of HSA, but
an increase in the free fraction of PF was observed in a solution of
AAG.

5. PF and 5-OH-PF both displayed a high affinity, low capacity site
on AAG; however, PF also displayed a low affinity, high capacity site.
The affinity of PF and 5-OH-PF to the low affinity, high capacity site
on HSA were similar. The interaction of PF and 5-OH-PF with
lipoproteins is speculated to be due to solubilization rather than
"true" protein-binding, except in the case of PF binding with HDL,
which appeared to display saturable binding. The solubilization of PF
and 5-OH-PF was found to be greatest in the LDL, followed by HDL, and
then the VLDL complexes.

6. The mathematical characterization PF and 5-OH-PF binding in
simulated serum resulted in estimates consistently higher than that of
actual data. When the binding contribution of VLDL and LDL was
eliminated, the binding more closely resembled that observed in
control serum.
7. The uptake of 5-OH-PF by red blood cells was greater than that observed for PF. The ratio of drug concentration in the red blood cell/drug concentration in plasma was almost 5 fold greater for 5-OH-PF than PF.

From the above observations it would appear that the binding characteristics of PF and 5-OH-PF are similar, varying only in affinity of the high affinity binding site, when determined separately. When parent drug is added, the binding profile (proteins important in binding a drug) of 5-OH-PF is altered significantly.
6. REFERENCES


Chan G. L.-Y., Propafenone Pharmacokinetics: GLC-ECD Analysis; Metabolic Induction by Phenobarbital in Non-Smoking and Smoking Healthy Volunteers; Protein Binding; Pharmacodynamics in Patients, Ph.D. Thesis, The University of British Columbia, Vancouver, B.C., 1989c.


# APPENDIX 1. PROTEIN COMPOSITION OF NORMAL VOLUNTEERS FOR BINDING STUDY

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>TOTAL PROTEIN</th>
<th>ALPHA-1-ACID GLYCOPROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64.0 ± 1.9 mg/mL</td>
<td>0.54 mg/mL</td>
</tr>
<tr>
<td>2</td>
<td>61.8 ± 2.3 mg/mL</td>
<td>0.77 mg/mL</td>
</tr>
<tr>
<td>3</td>
<td>67.0 --- mg/mL</td>
<td>0.62 mg/mL</td>
</tr>
<tr>
<td>4</td>
<td>58.0 ± 5.1 mg/mL</td>
<td>0.51 mg/mL</td>
</tr>
<tr>
<td>5</td>
<td>57.6 ± 0.3 mg/mL</td>
<td>0.58 mg/mL</td>
</tr>
</tbody>
</table>
APPENDIX 2. PROTEIN CONTENT (TOTAL PROTEIN AND α-1-ACID GLYCOPROTEIN) IN SOLUTIONS ISOLATED PROTEINS USED IN BINDING EXPERIMENTS.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TOTAL PROTEIN</th>
<th>ALPHA-1-ACID GLYCOPROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>FREE FATTY ACID FREE ALBUMIN</td>
<td>35.8 mg/mL</td>
<td>--</td>
</tr>
<tr>
<td>ALBUMIN IN ULTRAFILTRATE</td>
<td>40.0 mg/mL</td>
<td>--</td>
</tr>
<tr>
<td>ALBUMIN IN BUFFER</td>
<td>39.0 mg/mL</td>
<td>--</td>
</tr>
<tr>
<td>ALPHA-1-ACID GLYCOPROTEIN AND ALBUMIN IN BUFFER</td>
<td>39.0 mg/mL</td>
<td>0.68 mg/mL</td>
</tr>
<tr>
<td>ALPHA-1-ACID GLYCOPROTEIN AND ALBUMIN IN ULTRAFILTRATE</td>
<td>39.1 mg/mL</td>
<td>0.71 mg/mL</td>
</tr>
<tr>
<td>LIPOPROTEIN DEFICIENT SERUM</td>
<td>36.5 mg/mL</td>
<td>0.90 mg/mL</td>
</tr>
<tr>
<td>SERUM</td>
<td>46.8 mg/mL</td>
<td>0.69 mg/mL</td>
</tr>
<tr>
<td>ALPHA-1-ACID GLYCOPROTEIN IN BUFFER</td>
<td>--</td>
<td>0.71 mg/mL</td>
</tr>
<tr>
<td>ALPHA-1-ACID GLYCOPROTEIN IN ULTRAFILTRATE</td>
<td>--</td>
<td>0.67 mg/mL</td>
</tr>
</tbody>
</table>
## APPENDIX 3. COMPOSITION OF HUMAN SERUM ULTRAFILTRATE

<table>
<thead>
<tr>
<th>Test</th>
<th>Pre-filtration</th>
<th>Post-filtration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Protein</strong> (g/L)</td>
<td>81.0 ± 4.9</td>
<td>**</td>
</tr>
<tr>
<td><strong>Albumin</strong> (g/L)</td>
<td>47.2 ± 1.1</td>
<td>**</td>
</tr>
<tr>
<td><strong>Bilirubin</strong> (umol/L)</td>
<td>3.2 ± 0.8</td>
<td>**</td>
</tr>
<tr>
<td>Na⁺ (mmol/L)</td>
<td>140 ± 1.0</td>
<td>130 ± 11</td>
</tr>
<tr>
<td>K⁺ (mmol/L)</td>
<td>4.2 ± 0.1</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Cl⁻ (mmol/L)</td>
<td>104 ± 1</td>
<td>98 ± 6</td>
</tr>
<tr>
<td>CO₂ (mmol/L)</td>
<td>23 ± 1</td>
<td>18.6 ± 3.3</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>4.8 ± 1.1</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>98 ± 4</td>
<td>81.9 ± 12.6</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.5 ± 1.0</td>
<td>5.0 ± 1.0</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>5.97 ± 1.17</td>
<td>**</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>2.15 ± 1.59</td>
<td>0.07 ± 0.05</td>
</tr>
</tbody>
</table>

** NOT DETECTABLE
APPENDIX 5. PROTEIN CONCENTRATIONS IN SOLUTIONS OF ISOLATED HUMAN SERUM PROTEINS (AAG, HSA, HDL, LDL, AND VLDL) USED IN EXPERIMENTS IN WHICH BINDING WAS CHARACTERIZED.

<table>
<thead>
<tr>
<th>PROTEIN CLASS</th>
<th>PROTEIN</th>
<th>CONCENTRATION (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALBUMIN</strong></td>
<td><strong>SERUM</strong></td>
<td>40.9</td>
</tr>
<tr>
<td><strong>HIGH DENSITY LIPOPROTEINS</strong></td>
<td></td>
<td>48.9</td>
</tr>
<tr>
<td><strong>LOW DENSITY LIPOPROTEINS</strong></td>
<td></td>
<td>1.72</td>
</tr>
<tr>
<td><strong>VERY LOW DENSITY LIPOPROTEINS</strong></td>
<td></td>
<td>0.84</td>
</tr>
<tr>
<td><strong>ALPHA-1-ACID GLYCOPROTEIN</strong></td>
<td><strong>SERUM</strong></td>
<td>0.09</td>
</tr>
<tr>
<td><strong>ALPHA-1-ACID GLYCOPROTEIN</strong></td>
<td><strong>GLYCOPROTEIN</strong></td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td><strong>GLYCOPROTEIN</strong></td>
<td>0.85</td>
</tr>
</tbody>
</table>
APPENDIX 6: Agarose electrophoresis of lipoproteins before (c) and after equilibrium dialysis (1-6).